

INSTRUCTIONS
(a) If Convention application insert "Convention"

AHP-8643

59 6 3 6 9

AUSTRALIA

Patents Act

(a) Convention
LOCATED AT SUB-OFFICE

13 AUG 1986

Melbourne

(b) Delete one

APPLICATION FOR A STANDARD/~~REXX~~ (b) PATENT

61102/86

(c) Insert FULL name(s) of applicant(s)

4/We (c) American Home Products Corporation

(d) Insert FULL address(es) of applicant(s)

of (d) 685 Third Avenue, New York, New York 10017,
United States of America

(e) Delete one

hereby apply for the grant of a (e) Standard/~~REXX~~ Patent for an invention entitled

(f) Insert TITLE of invention

(f)

POLY-KRINGLE PLASMINOGEN ACTIVATOR

(g) Insert "complete"
OR "provisional"
OR "petty patent"

which is described in the accompanying (g) complete specification.

ACCEPTED AND AMENDMENTS

ALLOWED 21-2-90

(Note: The following applies only to Convention applications)

Details of basic application(s)

(h) Insert number, country and filing date for the/or EACH basic application

(h)	Application No.	Country	Filing Date
	766,163	United States of America	14th August, 1985
	884835	United States of America	11 July, 1986

Address for Service:

PHILLIPS ORMONDE AND FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne, Australia 3000

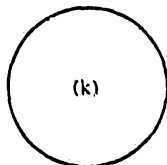
(i) Insert DATE of signing

Dated (i) July 18, 1986

(j) Signature of applicant(s) (for body corporate see headnote*)

(j) American Home Products Corporation

(k) Corporate seal if any



Note: No legalization or other witness required

George DeMott - Vice President

PHILLIPS ORMONDE AND FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne, Australia

Patents Act

DECLARATION FOR A PATENT APPLICATION

▼ INSTRUCTIONS

(a) Insert "Convention" if applicable
(b) Insert FULL name(s) of applicant(s)

In support of the (a) Convention application made by
(b) American Home Products Corporation

(c) Insert "of addition" if applicable
(d) Insert TITLE of invention

(hereinafter called "applicant(s)" for a patent (c) for an
invention entitled (d)

POLY-KRINGLE PLASMINOGEN ACTIVATOR

(e) Insert FULL name(s) AND address(es) of declarant(s) (See headnote*)

I/We (e) George DeMott, Vice President
American Home Products Corporation of
685 Third Avenue, New York, New York 10017,
United States of America

do solemnly and sincerely declare as follows:

~~XXXXXX I am/we are the applicant(s)~~
~~(or, in the case of an application by a body corporate)~~

1. I am/~~We are~~ authorized to make this declaration on behalf of the applicant(s).
~~XXXXXX I am/we are the actual inventor(s) of the invention.~~
~~(or, where the applicant(s) is/are not the actual inventor(s))~~

(f) Insert FULL name(s) AND address(es) of actual inventor(s)

2. (f) Paul Porwen Hung - 506 Ramblewood Drive, Bryn Mawr, Delaware County,
Pennsylvania, United States of America.

Narender Kumar Kalyan - 304A Murray Drive, King of Prussia,
Montgomery County, Pennsylvania, United States of America.

(g) Recite how applicant(s) derive(s) title from actual inventor(s) (See headnote**)

Shawguang Lin Lee - 155 Sth Spring Mill Rd., Villanova, Delaware County, Pennsylvania
is/are the actual inventor(s) of the invention and the facts upon which the applicant(s) USA.
is/are entitled to make the application are as follows:

(g)

Applicants are the assignees of the invention from the actual inventors.

(Note: Paragraphs 3 and 4 apply only to Convention applications)

(h) Insert country, filing date, and basic applicant(s) for the/or EACH basic application

3. The basic application(s) for patent or similar protection on which the application is based is/are identified by country, filing date, and basic applicant(s) as follows:

(h) United States of America filed 14th August, 1985 by
P.P. Hung, N.K. Kalyan and S.L. Lee.
United States of America filed 11 July, 1986 by
P.P. Hung, N.K. Kalyan and S.L. Lee

4. The basic application(s) referred to in paragraph 3 hercof was/were the first application(s) made in a Convention country in respect of the invention the subject of the application.

(k) Insert PLACE of signing

Declared at (k) New York, New York 10017,
United States of America

(l) Insert DATE of signing

Dated (l) July 18, 1986

(m) Signature(s) of declarant(s)

(m) American Home Products Corporation

Note: No legalization or other witness required

George DeMott
George DeMott
Vice President

To: The Commissioner of Patents

(12) PATENT ABRIDGMENT (11) Document No. AU-B-61102/86
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 596369

(54) Title
POLY-KRINGLE PLASMINOGEN ACTIVATOR

International Patent Classification(s)
(51)⁴ C12P 019/34 A61K 037/02 C07G 017/00 C07H 021/04
C12N 005/10 C12N 009/64 C12N 015/00 C12N 015/58
C12P 021/02

(21) Application No. : 61102/86

(22) Application Date : 13.08.86

(30) Priority Data

(31) Number	(32) Date	(33) Country
766163	14.08.85	US UNITED STATES OF AMERICA
884835	11.07.86	US UNITED STATES OF AMERICA

(43) Publication Date : 19.02.87

(44) Publication Date of Accepted Application : 03.05.90

(71) Applicant(s)
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(72) Inventor(s)
PAUL PORWEN HUNG; NARENDER KUMAR KALYAN; SHAWGUANG LIN LEE

(74) Attorney or Agent
PHILLIPS,ORMONDE & FITZPATRICK

(56) Prior Art Documents
AU 563031 14142/83 C12N 15/00

(57) C In accordance with this invention there is provided a group of hybrid, third generation, plasminogen activators containing plural polypeptide kringles; at least one of said kringles being heterologous and at least one being homologous, adjacent homologous and heterologous kringles being separated by an amino acid linker sequence. The polypeptides of this invention contain from 2 to 6 or more kringles eg 3 or 4 kringles. By heterologous kringles as used herein and in the claims applicants mean the polypeptide product contains at least one kringle corresponding to that found in a different, naturally-occurring source or any additional kringle structure common to and in addition to those found in a native plasminogen activator. It is understood that where a common kringle structure is to be added to a native plasminogen activator to produce

the hybrid plasminogen activators of this invention, the DNA is chemically synthesised and the DNA codon usage for production of that common kringle must differ from that found in the DNA coding sequence for the native plasminogen activator to avoid recombination (looping out) while generating the desired amino acid sequence of the native kringle. The kringles present in the hybrid plasminogen activators of this invention, although separately sharing areas of homology, are heterologous in nature and as a result thereof, the plasminogen activators of this invention differ in their kringle combination from any found in native plasminogen activators, either in amino acid sequence, number and/or size. In addition, this invention provides the genes coding for the hybrid plasminogen activators and key fragments thereof, expression vectors for DNA production of the complete polypeptides as well as key fragments thereof, microorganisms or cell cultures transformed with the expression vectors, compositions comprising the plasminogen activators and a method for using the hybrid plasminogen activators.

. A kringle is a triple looped polypeptide structure formed by three disulfide bonds. Kringles vary in length from about 79 to 82 amino acid groups. A high degree of sequence homology is shared among the single kringle of human urokinase (Gunzler et al., Hoppe-Seyler's Z. Physiol. Chem. 363, 1155, 1982), the two kringles of human tissue plasminogen activator (Pennica, et al. Nature, 301, 214, 1983), the two kringles of human prothrombin (Walz et al., Proc. Nat'l. Acad. Sci. USA., 74, 1969, 1977), and the five kringles of human plasminogen (Sottrup-Jensen et al., in Progress in Chemical Fibrinolysis and Thrombolysis (eds. Davidson et al), 3, 191, 1978). The relative positions of the six cysteins involved in the intra-kringle disulfide bridges are conserved in all kringles. The term, kringle(s) used herein and in the claims may be taken to mean any of such structure(s) in the above

mentioned proteins. It is also understood that polymorphic forms in the kringle region of these proteins may exist in nature where one or more amino acids may be added, deleted or substituted. Similar changes in a protein may also be brought about in vitro with the advent of the present day technology of point mutation of the gene or by chemical synthesis of the gene with any desired sequence. These modified structure(s) are therefore also included in the term, kringle(s), used in this application.

Preferably the polykringle plasminogen activator comprises the two kringles of t-PA, i.e. the polypeptide dequence corresponding to the amino acid sequence 92 to 261 of t-PA. Other useful kringle portions are amino acid sequences 1 to 261 t-PA; 92 to 527 of t-PA; 1 to 131 of urokinase, 50 to 131 of urokinase; 65 to 248 of prothrombin.

CLAIM

1. A hybrid plasminogen activator containing plural polypeptide kringles, at least one of said kringles being heterologous and at least one being homologous; adjacent homologous and heterologous kringles being separated by an amino acid linker sequence.

6. A plasminogen activator according to any one of Claims 1 to 4 containing a polypeptide sequence corresponding to one of the following amino acid sequences: 1 to 261 of t-PA,

92 to 527 of t-PA,

1 to 131 of urokinase,

50 to 131 of urokinase, or 65 to 248 of prothrombin.

(11) AU-B-61102/88
(10) 596369

-4-

17. A process for preparing a plasminogen activator according to any one of Claims 1 to 12 which comprises constructing by recombinant DNA techniques, a replicable expression vector containing the DNA sequence encoding for said hybrid plasminogen activator, transforming suitable host cells to introduce said expression vector and obtain transformed host cells, culturing said transformants to express said hybrid plasminogen activator and recovering said hybrid plasminogen activator.

AUSTRALIA

596369

Patents Act

COMPLETE SPECIFICATION

(ORIGINAL)

Class

Int. Class

Application Number: 61102/86

Lodged:

Complete Specification Lodged:

Accepted:

Published:

Priority

Related Art:

This document contains the
amendment made under
Section 49 and is correct for
printing.

APPLICANT'S REF.: AHP-8643/C2

Name(s) of Applicant(s):

AMERICAN HOME PRODUCTS CORPORATION

Address(es) of Applicant(s):

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NARENDER K KALYAN
SHAWGUANG LIN LEE

Address for Service is:

PHILLIPS, ORMONDE AND FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne, Australia, 3000

Complete Specification for the invention entitled:

POLY-KRINGLE PLASMINOGEN ACTIVATOR

The following statement is a full description of this invention, including the best method of performing it known to applicant(s):

- 2 -

This invention relates to plasminogen activators, more particularly to polykringle plasminogen activators to the means and methods for preparing such activators and to compositions containing them and to methods of using them.

Plasminogen activators are a class of serine proteases that convert plasminogen into plasmin. Plasmin degrades the fibrin matrix of blood clots, thereby restoring the hemodynamic condition of an open vascular system after an internal vascular accident has produced thrombosis or thromboembolism. Vascular disease states involving partial or total blockage of blood vessels which are amenable to treatment with plasminogen activators include stroke, pulmonary embolism, myocardial infarction, as well as deep vein and peripheral artery obstructions.

There are two immunologically distinct types of plasminogen activators found in human plasma and other body fluids - the urokinase-type plasminogen activator (u-PA; M_r , 55,000) and the tissue-type plasminogen activator (t-PA; M_r , 68,000). The activity of the tissue-type plasminogen activator is potentiated by fibrin. The enzyme acts at the site of a thrombus and demonstrates a higher affinity for fibrin than does the urokinase-type plasminogen activator (Haylaeris *et al.*, J. Biol. Chem., 257, 2912, 1982). Therefore, the tissue-type plasminogen activator is considered to be the physiologically relevant thrombolytic agent.

Both activators, u-PA and t-PA, share the following common features: 1) they are synthesised as single chain proenzymes which can be cleaved by plasmin or trypsin, without disrupting their disulfide linked two-chain molecular structure. Upon reduction, each plasminogen activator breaks down into a heavy and a light chain (M_r 33,000 for u-PA; M_r 35,000 for t-PA); 2) both enzymes are serine proteases which can be inactivated by

- 3 -

serine-specific reagents such as diisopropyl fluorophosphate; and 3) both enzymes contain a triple disulfide-linked sequence of amino acids which form a loop or kringle in the molecule. Urokinase plasminogen activator has a single kringle. Tissue plasminogen activator has two kringles connected by a hexapeptide linker sequence. These kringles are believed to be responsible for binding of the enzymes to fibrin (Thorsen, Biochem. Biophys. Acta., 393, 55, 1975).

The DNA sequence analysis and the amino acid sequence of t-PA is disclosed by Pennica et al., Nature, 301, 214, (1983), Ny et al., Proc. Natl. Acad. Sci. USA 81 5355 (1984) and European Patent Application 93, 619 to Genentech Inc. The DNA sequence analysis and the amino acid sequence of u-PA is disclosed in European Patent Application 92, 182 to Genentech Inc. and urokinase cDNA is discussed by Verde et al., Proc. Natl. Acad. Sci. USA 81 4727 (1984).

In accordance with this invention there is provided a group of hybrid, third generation, plasminogen activators containing plural polypeptide kringles; at least one of said kringles being heterologous and at least one being homologous, adjacent homologous and heterologous kringles being separated by an amino acid linker sequence. The polypeptides of this invention contain from 2 to 6 or more kringles eg 3 or 4 kringles. By heterologous kringles as used herein and in the claims applicants mean the polypeptide product



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1



- 4 -

kringles present in the hybrid plasminogen activators of this invention, although separately sharing areas of homology, are heterologous in nature and as a result thereof, the plasminogen activators of this invention

5 differ in their kringle combination from any found in native plasminogen activators, either in amino acid sequence, number and/or size. In addition, this invention provides the genes coding for the hybrid plasminogen

10 activators and key fragments thereof, expression vectors for DNA production of the complete polypeptides as well as key fragments thereof, microorganisms or cell cultures transformed with the expression vectors, compositions comprising the plasminogen activators and a method for using the hybrid plasminogen activators.

15 A kringle is a triple looped polypeptide structure formed by three disulfide bonds. Kringles vary in length from about 79 to 82 amino acid groups. A high degree of sequence homology is shared among the single kringle of human urokinase (Gunzler et al., Hoppe-Seyler's

20 Z. Physiol. Chem. 363, 1155, 1982), the two kringles of human tissue plasminogen activator (Pennica, et al. Nature, 301, 214, 1983), the two kringles of human prothrombin (Walz et al., Proc. Nat'l. Acad. Sci. USA., 74, 1969, 1977), and the five kringles of human plasminogen

25 (Sottrup-Jensen et al., in Progress in Chemical Fibrinolysis and Thrombolysis (eds. Davidson et al), 3, 191, 1978). The relative positions of the six cysteins involved in the intra-kringle disulfide bridges are conserved in all kringles. The term, kringle(s) used herein and in the claims

30 may be taken to mean any of such structure(s) in the above mentioned proteins. It is also understood that polymorphic forms in the kringle region of these proteins may exist in nature where one or more amino acids may be added, deleted or substituted. Similar changes in a protein may also be brought about in vitro with the advent of the present day technology of point mutation of the gene or by chemical

- 5 -

synthesis of the gene with any desired sequence. These modified structure(s) are therefore also included in the term, kringle(s), used in this application.

Preferably the polykringle plasminogen activator
5 comprises the two kringles of t-PA, i.e. the polypeptide
dequence corresponding to the amino acid sequence 92 to
261 of t-PA. Other useful kringle portions are amino
acid sequences 1 to 261 t-PA; 92 to 527 of t-PA;
1 to 131 of urokinase, 50 to 131 of urokinase; 65 to 248
10 of prothrombin.

The following description specifically illustrates the
production of a tris-kringle plasminogen
activator, as well as a tetra-kringle plasminogen activator.
The methods employed are representative of those applicable
15 to the production of the other polypeptides of this invention.
Tris-kringle as used herein and in the claims means a triple kringle

The tris-kringle plasminogen activators of this
invention, as constructed by recombinant DNA techniques from
appropriate genetic coding sequences of urokinase and
20 t-PA clones, offer the advantages of increased stability,
increased binding affinity for fibrin and improved half-
life in vivo when compared to either of the native
plasminogen activators. These properties of the tris-
kringle plasminogen activators provide improved biological
25 potency and improved shelf life. The tris-kringle-PA
molecule is easier to handle during production and
purification than native t-PA because the latter polypeptide,
as found in culture fluids and various purification stages,
is accompanied by low molecular weight, heavy and light
30 chain fragments. The tetra-kringle plasminogen activator
as well as the other poly-kringle plasminogen activators
share these properties.

The tris-kringle plasminogen activator of this
invention is constructed by combining the N-terminal portion
of urokinase through its single kringle region with that

- 6 -

portion of t-PA beginning at or before the beginning of the double kringle via a suitable linker. The single kringle of urokinase is known to precede the glycine residue at amino acid position number 131. The double kringle of t-PA is known to begin at the cysteine following a threonine residue at position number 91. Thus, the N-terminal portion of urokinase terminated at the glycine residue at number 131 is joined, optionally through a suitable linker mimicking the hexapeptide link between the two kringles of t-PA, to a t-PA molecule from which the first 91 N-terminal amino acids have been deleted. The resulting hybrid molecule is larger than t-PA by 46 amino acid residues providing a protein of about 73,000 M.W. (t-PA has a molecular weight of 68,000) depending upon the specific linker employed. Similarly, the single kringle of urokinase (UKKaa50-131), in conjunction with a kringle linker, is inserted into the t-PA polymer between amino acids 91-92 or 261-262 to afford two different genes for production of tris-kringle plasminogen activators. Similarly, and by standard techniques, the u-PA kringle may be inserted, with appropriate linkers, between the two t-PA kringles. In addition, the kringles found in plasminogen or prothrombin may be isolated and inserted in any of the position of t-PA mentioned above. The construction of any of the poly-kringle plasminogen activators of this invention follows the same basic plan of isolating single or double kringle region from the protein mentioned and inserting them into the backbone of the t-PA molecule.

Kringle linkers employed in this invention may have 6 to 20 amino acid moieties, preferably 6 to 10, e.g. L-Ser-L-Glu-Gly-L-Asn-L-Ser-L-Asp, or L-Thr-L-Asp-L-Ala-L-Glu-L-Thr-L-Glu or combinations of such amino acids.

The kringle linkers employed to join the single kringle portion of urokinase (UKK) to the double kringle region of t-PA (t-PK1 and t-PK2) is a polypeptide containing 6 to 10 natural amino acid moieties. Preferably

- 7 -

the kringle linker is selected to maintain a similar spatial arrangement to that which exists between the two kringles of t-PA. As such, the preferred linker is L-Ser-L-Glu-Gly-L-Asn-L-Ser-L-Asp because it is identical to that joining t-PK1 to t-PK2 in t-PA.

5 However, the hexapeptide L-Thr-L-Asp-L-Ala-L-Glu-L-Thr-L-Glu represents another applicable hexapeptide linker and any combination of L-Ala, Gly, L-Ser, L-Glu, L-Thr and L-Asp may be employed on the N-terminal or C-terminal ends of the hexapeptide linker to provide a more open structure as a hepta- ,
 10 octa-, nona- or decapeptide link between the t-PA kringles and the UK kringle. Other linkers will be obvious to the chemist. For simplicity, the kringle linker specifically mentioned throughout the rest of this application is limited to the preferred linker referred to above.

15 The tris-kringle plasminogen activators are produced by limited digestion of urokinase and t-PA coding sequences with selected restriction enzymes to afford the desired u-PA and t-PA fragments. The fragments are isolated by fractionation on agarose or acrylamide gels, ligated together and introduced into an appropriate vector
 20 or vectors for cloning and subsequent expression.

Plasminogen activators produced by this invention include
 91(PTK⁶⁵⁻²⁴⁸ Ser-Glu-Gly-Asn-Ser-Asp)-92-t-PA;
 (UKaa¹⁻¹³¹-Kringlelinker)¹⁻⁹¹ t-PA; 91-(UKaa⁵⁰⁻¹³¹- kringle linker) -
 92t-PA; 261-(Kringlelinker - UKaa⁵⁰⁻¹³¹) 262 t-PA in which the Kringle-
 25 linker is a polypeptide moiety containing 6 to 10 natural amino acids e.g. Ser-Glu-Gly-Asn-Ser-Asp.

This invention provides a process for preparing a plasminogen activator having plural heterologous kringles culturing a host cell which has been transformed with recombinant DNA coding for said
 30 plasminogen activator. Also provided by this invention is a process for preparing said polykringle.

- 8 -

plasminogen activators which comprises constructing by recombinant DNA techniques, a replicable expression vector containing the DNA sequence encoding for said hybrid plasminogen activator, transforming suitable host cells to introduce said expression vector and obtain transformed host cells, culturing said transformants to express said hybrid plasminogen activator and recovering said hybrid plasminogen activator.

Description of the Drawings

Figure 1 presents schematic configurational drawings of three triskringle plasminogen activators and one tetra-kringle plasminogen activator produced by the methods disclosed in this application. The darkened portion of the depicted structures 1 (a-c) defines that portion of urokinase containing the urokinase kringle (UKK).

The darkened portion of structure 1(d) defines the double kringle region of prothrombin (PTK1 and PTK2). The abbreviations tPK1 and tPK2 define the tissue plasminogen activator kringle 1 and 2, respectively.

Figure 2 depicts the restriction map of tissue plasminogen activator recombinant clone pWP-42.

Figure 3 presents the technique followed in production of plasmid ptPBM-1 from pWP-42. ptPBM-1 contains the genetic information needed to produce the complete t-PA molecule.

Figure 4 presents the technique followed in production of plasmid pUKBM from plasmid pUK-53. pUKBM contains the genetic information needed to produce the complete urokinase protein.

Figure 5 presents a flow diagram of the method

- 9 -

followed to produce the gene coding for the tris-kringle product depicted in Figure 1(a).

Figure 6 presents a flow diagram of the method followed to produce the gene coding for amino acids 51-131 of urokinase (u-PA⁵¹⁻¹³¹).
5

Figure 7 presents a flow diagram of the method followed to produce the gene coding for the tris-kringle product depicted in Figure 1(b).

- 10 -

Figure 8 presents a flow diagram of the method followed to produce the gene coding for the tris-kringle product depicted in Figure 1(c).

Figure 9 presents the DNA sequence of the gene coding for the product of figure 1(a) with reference to the urokinase signal peptide region (20
5 amino acids), the UKK region (amino acids 1-131 of urokinase), the hexapeptide linker and the remaining portion of the t-PA molecule (amino acids 92-527).

Figure 10 presents the DNA sequence of the gene coding for the product of Figure 1(b) with reference to the t-PA signal peptide (35 amino acids) the N-terminal portion of t-PA (amino acids 1-91), the UKK (amino acids 50-131
10 of urokinase), the hexapeptide linker and the C-terminal portion of t-PA (amino acids 92-527).

Figure 11 presents the DNA sequence of the gene coding for the product of Figure 1(c) with reference to the t-PA signal peptide (35 amino acids), the N-terminal portion of t-PA (amino acids 1-261), the hexapeptide linker, the
15 UKK (amino acids 50-131 of urokinase) and the C-terminal portion of t-PA (amino acids 262-527).

Figure 12 presents a flow diagram of the method followed to produce the gene coding for the double kringle region of prothrombin.

Figure 13 presents a flow diagram of the method followed in
20 producing the gene coding for the tetra-kringle product depicted in Figure 1(d).

Figure 14 presents a BPV-I based expression vector system designed for expression of plasminogen activator Hybrid A (corresponding to Fig. 1(a)) and Hybrid B (corresponding to Fig. 1(b)) genes in mammalian cell line C-127 (mouse). The genes to be expressed, are inserted between the mouse metallo-
25 thionein transcriptional promoter element and the SV40 early region transcriptional processing signals.

Figure 15(a) presents screening of PA-producing cells or foci on a fibrin agar plate by applying 10 μ l of culture medium per well and incubating at 37°C till clear zones around the wells appear (lanes 2 to 7). Lane 1 shows the standard t-PA with enzyme concentration (units/ml) from top to bottom: 500, 250, 100, 50, 25 and 0.

Figure 15(b) presents a zymogram on a fibrin agar plate of Hybrid B, Hybrid A and tissue type-plasminogen activators after separation of the hybrid-PA's by electrophoresis in a polyacrylamide gel (PAGE).

Figure 15(c) presents an autoradiogram of 35 S-labelled plasminogen activators, Hybrid A and Hybrid B, after immunoprecipitation of radio-pulsed medium with anti-t-PA antiserum and then electrophoresis on non-reducing sodium dodecyl sulfate (SDS)/PAGE. Protein markers (right lane) of known molecular weights were run concurrently.

Methods and Materials

a) Enzymic Reactions: The restriction and DNA modifying enzymes were obtained from New England Biolabs Inc., Beverly, MA or International Biotechnologies Inc., New Haven, CT. A typical restriction enzyme reaction was performed in a total volume of 50 μ l following the procedure(s) recommended by the supplier of the enzyme.

A ligation reaction for the sticky end DNA is typically performed at 15°C overnight in a buffered 20 μ l solution containing 100-200 ng DNA and 400 units of T4 DNA ligase (N.E. Biolabs.). For blunt end ligation, 4 units of T4 RNA ligase (N.E. Biolabs.) are included in the above reaction mixture. (Goodman, H.M., and MacDonald, R.J., Method. Enzymol. 68, 75, 1979). The buffer solution used is prepared as a stock 10X solution; 0.5 M Tris⁺HCl (pH 7.6), 0.1 M MgCl₂ and 0.1 M DTT (dithiothreitol).

b) Synthesis of Oligonucleotides: All the oligonucleotides mentioned in this application were synthesized by the phosphotriester method (Crea et al., Proc. Nat'l. Acad. Sci. (USA) 75, 5765, 1978) using the Gene Machine model 380A (Applied Biosystems Inc., Foster city, CA). Before their use in ligation reactions, the oligomers were phosphorylated at the 5' end in a volume of 50 μ l containing 200-500 ng DNA, 10 units of T4 DNA kinase, 0.5 mM ATP and kinase buffer (0.05M Tris·HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT) and incubated at 37°C for 1/2 hour. For use as hybridization probes, oligomers were radiolabeled with 100 μ Ci gamma ³²P-ATP (5,000 Ci/mol, Amersham, Arlington Heights, IL) following the procedure of Maxam, A.M. and Gilbert, W. Method Enzymol. 65, 499 (1980).

c) Isolation of DNA Fragments DNA fragments were first separated by electrophoresis through 0.5-1.5% agarose gel. Electrophoresis is carried out at about 100 volts for 2-4 hours in Tris-Borate-EDTA (TBE) buffer (0.089M Tris, 0.089 M boric acid, 2 mM EDTA, pH 8.0). DNA bands are visualized under UV light by staining the gel in 0.5 μ g/ml ethidium bromide solution (Sharp et al. Biochem. 12, 3055, 1973). The agarose containing the DNA band is cut out with a razor. The DNA is electroeluted from the gel. (Maniatis et al. Molecular Cloning, a Laboratory Manual, p. 164, 1982). The DNA is further purified by passing it through an Elutip-d[®] column (Schleicher and Schuell, Keene, NH). The DNA is precipitated with ethanol. After centrifugation in an Eppendorf microfuge for 15 minutes, the pellet is washed once with 70% ethanol, dried under vacuum and dissolved in 50 μ l deionized water.

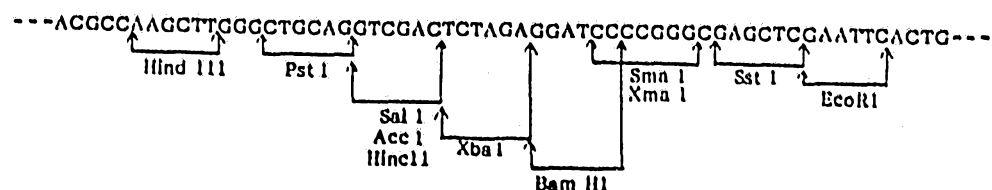
d) Miniplasmid DNA Preparation: About 2 ml of LB (Luria Bertani) medium containing appropriate antibiotics is inoculated with a single bacterial colony and is incubated at 37°C overnight with vigorous shaking. About 1.5 ml of the culture medium is used to isolate plasmid DNA by the boiling method

described in Maniatis et al., loc. cit. p. 366. The rest of the culture is stored in 15% glycerol at -20°C . for later use. The DNA is dissolved in $40\ \mu\text{l}$ H_2O containing $10\ \mu\text{g}$ RNase /ml. About $8\ \mu\text{l}$ is sufficient for one restriction enzyme analysis.

- 5 e) Large Scale Preparation of Plasmid DNA: Typically, one liter of LB medium is inoculated with a single bacterial colony. After amplification of the plasmid DNA with chloramphenicol, the bacterial cells are harvested and lysed according to the boiling method (Holmes, D.S. and Quigley, M. Anal Biochem. 114, 193, 1981). The plasmid DNA is further purified either by cesium
10 chloride gradient centrifugation or by column chromatography on a Sepharose 4B column (Pharmacia, Uppsala, Sweden) as described in Maniatis et al., loc. cit. pp. 93-96. A recovery of about $400\ \mu\text{g}$ DNA per liter culture is routinely obtained.

- f) Vectors: dG-tailed pBR322 plasmid DNA (Bethesda Research Laboratories, Inc., Gaithersburg, MD) was used to clone the cDNA for t-PA and
15 u-PA. The detailed molecular structure of pBR322 is described by Maniatis et al., loc. cit. pp. 5 and 488. The E. coli strains used for transformation with recombinant pBR322 were either HB101 or MM294 (Maniatis et al., loc. cit. p. 504).

- All of the subcloning of DNA fragments from t-PA and u-PA genes
20 were performed in pUC plasmids - a series of pBR322 derived vectors containing lac Z and ampicillinase genes (Vieria, J. and Messing, J., Gene 19, 259, 1982). In addition the plasmids also contain a sequence - multiple cloning or restriction site - in the lac Z, as shown below:



Cloning in any of the 11 sites can be monitored by the appearance of white recombinant colonies in the background of blue vector colonies on an indicator plate containing X-gal (5-bromo-4-Chloro-3-indolyl β -D-galactoside) (Ruther, Mol. Gen. Genetics 178, 475, 1980). The E. coli strain used for transformation
5 with the recombinant pUC plasmid, was JM 103. The pUC plasmid and E. coli JM 103 were obtained from Pharmacia P-L Biochemicals, Milwaukee, WI.

g) Host/vector System

1. Microbial System

The work described here was performed using the microorganisms E. coli. K-12 strain JM 103 (PL Biochemicals) and E. coli K-12 strain MM294
10 (ATCC No. 33625). Other microorganisms which may be used in this process include other useful E. coli strains and Bacilli, such as Bacillus subtilis. All these microorganisms utilize plasmids that can replicate and express heterologous gene sequences.

15 The expression system in yeast employs a plasmid which is capable of selection and replication in E. coli and/or yeast (*Saccharomyces cerevisiae*). For selection in yeast, the plasmid contains the TRP 1 gene which renders a transformed trp^- yeast strain (RH218) prototrophic for tryptophan. The yeast expression vector can shuttle in between yeast and E. coli. The plasmid has the
20 following components: 1) a DNA segment derived from PBR 322 containing the origin of replication and the ampicillin resistance gene, 2) the yeast TRP 1 gene, 3) the yeast 2 μ DNA which enables the plasmid to replicate in yeast with high stability, 4) A promoter region from the yeast gene, such as alcohol dehydrogenase, α factor, glyceraldehyde-3-phosphate-dehydrogenase, etc., 5) transla-
25 tional start and transcriptional stop sequences which can be used for proper termination and polyadenylation of mRNA in the expression system.

2. Mammalian Cell Culture System

Mammalian cell lines capable of the replication and expression of a compatible vector for the production of heterologous proteins can be used in the present invention. They are, for example: Cos-7, WI38, 3T3, CHO, Hela cells, 5 and C127 cells. The vectors used contain 1) the origin of replication derived from a virus (SV40, adeno, polyoma, BPV) or cellular chromosomal DNA, 2) a promoter, 3) the translational initiation signals, such as ribosomal binding sites, and 4) RNA processing signals, (RNA splicing, polyadenylation and transcriptional terminator sequences). Specific examples of the expression vectors 10 presented here use a BPV viral replication origin, a mouse metallothionein promoter and SV40 RNA processing signals. The vector can also be shuttled between mammalian cell culture and E. coli. It contains derivatives of PBR 322 sequences which provide selectable markers for E. coli ampicillin resistance as well as an E. coli origin of DNA replication. These sequences are derived from 15 the plasmid pML-2d.

The edited hybrid plasminogen activator gene containing a Bam HI sticky end is first inserted at the Bgl II site of plasmid 341-3 (Law MF et al., Md. Cell Biol. F 3, 2110, 1983) between the mouse metallothionein transcriptional promoter element and the SV40 early region transcriptional processing signals. 20 The complete BPV genome, obtained after digestion of plasmid 142-6 (ATCC No.37134) with Bam HI, is ligated to the unique Bam HI site. Plasmid 341-3 also contains pML2, a pBR 322 derivative which allows plasmid replication in bacterial cells. The expression plasmid constructed herein can replicate in mouse C-127 cells exclusively as an extrachromosomal episome. Transfected 25 cells can be selected for the transformed phenotype. Further modification of the expression vector, such as by adding specific enhancer elements for higher

expression levels or inserting drug resistance (such as neomycin resistance) into the gene is also possible.

TRIS-KRINGLE PLASMINOGEN ACTIVATOR

Tissue Plasminogen Activator (t-PA)

Messenger RNA

5 Total RNA was isolated by the isothiocyanate method (Maniatis et al., loc. cit. p. 196) from normal human fibroblast cells (WI-38 cells), which had been stimulated by endothelial cell growth factor (ECGF) and heparin to produce t-PA. The same stimulated cells produce urokinase. Messenger RNA (mRNA)
10 was obtained from the total RNA by chromatography on an oligo-deoxythymidine (dT)-cellulose column (Aviv et al., Proc. Natl. Acad. Sci. USA, 69, 1408, 1972). Further fractionation of the mRNA was performed by centrifugation in a 15-30% sucrose density gradient and individual mRNA fractions were hybridized with ³²P-probes. (as described below). Fractions containing the t-PA message (ca.
15 20-24S) were pooled for use in the preparation of complementary DNA (cDNA).

Complementary DNA

The pooled mRNA (5 µg) described in the previous paragraph was used to produce double stranded cDNA and the cDNA was homopolymer tailed with polydeoxycytidylate (poly dC) using terminal nucleotide transferase. The
20 product was annealed with Pst I digested, polydeoxyguanylate (poly dG) tailed pBR322. The annealed DNA was used to transform competent E. coli 294 cells which were cultured to produce about 10⁵ bacterial clones (Maniatis et al., loc cit., p. 229).

Screening and Identification of t-PA Clone

The following three oligonucleotides, after radiolabeling with ^{32}P -ATP, were used to screen the library of recombinant clones. These oligomers correspond to amino acid sequences, 34-39 (17 mer), 253-258 (18 mer) and 523-527 (15 mer) of t-PA molecule (Pennica, D. et al., Nature, 301 214, 1983) 17 mer: 5'-CCACTGTTGCACCAGCA-3'; 18 mer: 5'-CACATCACAGTACTCCCA-3'; 15 mer: 5'-CGGTCGCATGTTGTC-3'. About 20 colonies exhibited moderate to strong homology with the pooled probes. Replating and rehybridization of these colonies gave 16 clones with positive signals. Plasmid DNA prepared from these clones was blotted on nitrocellulose paper and hybridized with individual probes. Two clones (42 and 62a) hybridized to both the middle (18 mer) and 3' end (15 mer) probes. Enzymatic digestion of plasmid DNA with Pst 1 showed that clone No. 42 contained the biggest insert of greater than 2 kilobase (Kb) in the form of three fragments of 1.1, 0.6 and 0.4 Kb. A restriction map of the clone (pWP 42) is depicted in Figure 2 of the drawings. This clone contains the full length sequence for the t-PA gene, containing 2600 bp, which includes the 5'- and 3'- untranslated regions.

Editing of t-PA Gene

Approximately 10 μg of pWP 42 plasmid DNA was digested with 9 units Xho II at 37°C . for 2 hours. The reaction mixture was run on preparative 1.2% agarose gel and a 1618 bp DNA fragment was isolated by electrophoresis in agarose gel. After filling in cohesive ends with *E. coli* Polymerase I (Klenow fragment) and dNTPs (four deoxy nucleotide triphosphates- dATP, dGTP, dCTP and dTTP) 1 μg of the so modified DNA was ligated overnight with 300 ng of phosphorylated Sal I linker. After phenol/chloroform extraction and ethanol precipitation, the DNA was digested with 50 U of Sal I for four hours and the



- 18 -

reaction mixture applied to a preparative 1% agarose gel to isolate the desired DNA fragment.

The DNA with Sal I ends was ligated to Sal I cut pUC 13 and used to transform E. coli JM 103 cells and the cells were plated out on ampicillin and X-gal plates. Eight ampicillin resistant, white colonies were selected and grown to prepare a mini-plasmid preparation. Two clones (ptPS34B and ptPS39) were found to contain the required DNA fragment. Ten µg of ptPS39 plasmid DNA digested to completion with Bam HI and Nar I was run on preparative agarose gel to obtain a 1288 bp fragment coding for the C-terminal end of t-PA.

10 The 5' end of the t-PA gene was obtained by digestion of 10 µg of pWP 42 with four units of Hga I at 37°C. for eight hours. A 515 bp fragment was isolated by electrophoresis in 1% agarose gel. The cohesive ends of this DNA fragment were filled in with DNA polymerase I (Klenow fragment) and dNTPs and the product was ligated to Sma I cut pUC 13. After transforming E.
15 coli JM 103 cells, approximately 75 ampicillin resistant, white colonies were obtained. Twenty four of these colonies were grown to prepare a miniplasmid preparation. The miniplasmid preparation was digested with Nar I and 17 clones were found to have the required insert in either orientation. One clone (pt PHga 4) was grown in 1.0 liter of LB medium containing ampicillin to obtain a large
20 quantity of plasmid DNA using the boiling method. The plasmid DNA, pt PHga 4, was digested with Bam HI and Nar I and electrophoresed on 1.2% agarose gel to isolate a 434 bp DNA fragment coding for the N-terminal end of t-PA.

The 1288 bp DNA (300 ng) and 434 bp DNA (100 ng) were ligated overnight to obtain a 1722 bp DNA fragment. This DNA, after ligation with Bam
25 H I cut pUC 13 was used to transform E. coli JM 103 cells. More than 1000 ampicillin resistant colonies were obtained. Plasmid DNA from twelve colonies was prepared by the boiling method. The plasmid DNA was identified by cutting

- 19 -

with each of Bam H1, Nar 1 and Xho 11. All of the resulting plasmids were found to contain the desired 1,722 bp DNA fragment. One plasmid (pt PBM 1) was used for large scale plasmid DNA preparation. This plasmid, when cut with Bam H1, gave rise to the 1,722 bp DNA coding for the complete t-PA molecule. The
5 pt PBM 1 clone restriction map and a schematic diagram of its preparation is depicted in Figure 3.

Urokinase Plasminogen Activator (u-PA)

Screening and Identification of u-PA Clone

The library of 10^5 recombinant bacterial clones from which the t-PA
10 gene was derived, supra, was screened with a radiolabelled 18 mer probe by the method of Grunstein et al., Proc. Nat'l. Acad. Sci. USA, 72, 3961, (1975). The probe, synthesized by the standard phosphotriester method using a Gene Machine (Applied Biosystems), presents the oligomer sequence - 5'- GTA GAT GGC CGC AAA CCA - 3' -corresponding to the middle part of the urokinase gene (aa¹⁷³-
15 179). About 13 clones exhibited a moderate to strong hybridization signal. These clones were grown in 2 ml LB medium containing tetracycline and a miniplasmid preparation was prepared. The miniplasmid preparation was dissolved in 40 μ L H₂O containing 10 μ g/ml RNase. About 8 μ L of the DNA thereby produced was digested with one unit of Pst 1 and the product separated
20 by electrophoresis on 1% agarose gel. One clone (pUK 53) was found to contain the largest insert of 1.7 Kb in the form of three inserts of sizes 1.2, 0.4 and 0.1 Kb long. The complete 3'- end nucleotide sequence of urokinase was present in the Pst 1 cut 1.2 Kb DNA fragment. The 5' end sequence of the gene was discovered, through nucleotide sequencing by the Maxam and Gilbert method,
25 Methods Enzymol., 65, 1499, (1980) to be missing approximately 30 nucleotides corresponding to the first 10 amino acids of the signal peptide coding region of

- 20 -

the urokinase protein. Therefore, a duplex DNA sequence corresponding to the missing nucleotides was synthesized and ligated to the existing gene.

Editing of Urokinase Gene

5 The Urokinase plasmid (pUK 53) DNA was cut with Nco 1 and Mst II and the products separated by electrophoresis on 1% agarose gel. A DNA fragment of 1198 bp was isolated by electroelution. The 5' protruding end of the DNA
10 fragment corresponding to the Nco 1 cut was made blunt ended by filling in with dNTP's and E.coli DNA polymerase (Klenow fragment). The DNA was then ligated to Sma 1 cut pUC 13 and the modified plasmid used to transform competent E. coli JM 103 cells. The Nco 1 site of the
15 insert is regenerated when the DNA is ligated to the Sma 1 site of pUC 13. The cells were plated out on ampicillin and X-gal plates and a miniplasmid preparation produced from white colonies. Digestion of the miniplasmid DNA
20 preparation with Nco 1 and Sal 1 gave an approximate 1200 bp DNA fragment. A large scale plasmid DNA preparation from a positive clone (pUKNM-3') was made and digested with Nco 1 and Sal 1 to obtain a large amount of the
approximate 1200 bp DNA fragment which was separated by preparative agarose gel electrophoresis.

25 To provide the approximate 30 nucleotides corresponding to the first 10 amino acids of the 5' signal peptide coding region of the urokinase protein, pUK 53 plasmid DNA was digested first with Pst 1 and a 400 bp DNA fragment was isolated. This DNA was then treated with ScrF1 to yield a 242 bp fragment of DNA. The protruding ends of the DNA
30 were filled in with dNTP's and E.coli DNA polymerase 1 (Klenow fragment).

Two complementary oligonucleotide sequences, 38 and 42 bases in length, were synthesised on a Gene Machine to provide for missing amino acids (-9 to -20) while keeping

- 21 -

the proper translational reading frame and providing a Sal 1 sequence on both ends of the DNA for subcloning in Sal 1 cut pUC 13. The two oligomers were mixed in equimolar amounts in ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM dithiothreitol) and heated to 80°C. for 5 minutes and allowed to cool to room temperature for about 1 hour. The thus formed duplex of the two complementary nucleotide sequences (about 1 µg) was ligated to about 300 ng of the 242 bp DNA fragment in ligase buffer at 4°C for 16 hours using 400 units of T4 DNA ligase. The ligated mixture was separated by electrophoresis on 1.2% agarose gel and an approximate 320 bp DNA fragment was isolated by electroelution. This fragment (about 20 ng) was ligated to 100 ng. of Sal 1 cut pUC 13 and the vector was used to transform competent E. coli JM 103 cells. The cells were plated out on ampicillin X-gal plates. Twelve white colonies were selected and grown to prepare a miniplasmid preparation. The miniplasmid preparation was cut with Sal 1. One clone, containing the expected 320 bp DNA insert, was grown for large scale preparation of plasmid DNA. The DNA was cut with Sal 1 and Nco 1 to yield a 260 bp DNA fragment upon preparative agarose gel electrophoresis.

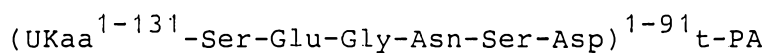
The 260 bp DNA and 1200 bp DNA fragments, containing a common Nco 1 restriction site at 333 bp position of the gene, were mixed in equimolar amounts for ligation. The ligated product was cut with Sal 1 and the reaction mixture separated by preparative 1% agarose gel electrophoresis. A 1460 bp DNA fragment was isolated by electroelution. This DNA was ligated to Sal 1 cut pUC 13 and this plasmid was used to transform competent E. coli JM 103 cells which were plated out on ampicillin and X-gal plates. Twelve white colonies were selected and grown to prepare a miniplasmid preparation by the boiling method. The miniplasmid preparation was cut with Sal 1 and one clone (pUKBM) was found to contain the desired 1460 bp DNA insert.

- 22 -

pUKBM was grown in large volume to provide plasmid DNA. The oligonucleotide sequence from the 5' end containing the synthetic linker was sequenced by the Maxam - Gilbert method to confirm its authenticity.

5 The DNA insert in pUKBM plasmid was thereby established to contain the translational initiation codon AUG (met, -20 aa in the leader sequence) as well as the termination codon TGA. This complete gene codes for the 20 amino acids of the signal peptide (-1 to -20) and the 411 amino acids of mature
10 urokinase protein.

Example 1



In compound (a.) shown in Fig.1, the t-PA sequence containing the signal peptide (a.a. -35 to -1) and the N-
15 terminal peptide (a.a. 1 to 91) regions were replaced by an amino acid sequence containing the signal peptide (a.a. -20 to -1) and the first 131 amino acids of urokinase. The u-PA sequence was joined to the first kringle (tPK1) of t-PA via
20 an oligonucleotide sequence coding for the hexapeptide L-Ser-L-Glu-Gly-L-Asn-L-Ser-L-Asp.

About 10µg of plasmid pUKBM was digested with Eco R1 and Mst 1 under standard conditions. The reaction mixture was electrophoresed through a 1.2% agarose gel at 150 volts for 3 hours. After staining the gel with ethidium bromide
25 to visualise the DNA bands, a 452 bp DNA fragment was isolated and purified. This DNA fragment contains coding information for the leader or signal peptide (20 amino acids) as well as N-terminal and kringle region (a.a. 1 to 131) of the urokinase gene.

30 In order to obtain the t-PA-des a.a. 1-91 sequence, about 10µg of recombinant plasmid ptPBM-1 was digested to completion with Ava II and electrophoresed on a 1.2% agarose gel to isolate a 747 bp DNA fragment. The

- 23 -

addition of an oligomer linker to this fragment followed by digestion with Eco R1 to obtain a 354 bp DNA fragment is described in Example 2 and depicted in Fig.7. The 354 bp DNA codes for the hexapeptide linker and the t-PA peptide (a.a. 92 to 204) - a 118 amino acid sequence. Equimolar amounts of the 452 bp DNA fragment prepared in the preceding paragraph and the 354 bp DNA fragment were ligated using T4 DNA ligase at 15°C for 16 hours. The reaction mixture was separated by electrophoresis on 1.2% agarose gel and a DNA band corresponding to a size of 806 bp was eluted out and purified. About 100 ng of 806 bp DNA fragment was ligated with about 300 ng of BAP (bacterial alkaline phosphatase) treated Eco R1 pUC 13 (Pharmacia P-L Biochemicals, Inc. Milwaukee, WI) in about 20µl reaction volume. About ¼ of the solution was used to transform competent E.coli JM 103 cells according to the procedure of Viera, J. and Messing, J., Gene 19, 259, (1982). Miniplasmid DNA prepared from 12 recombinant white colonies, was digested with Eco R1 under standard conditions. One clone, ptPUK-806, containing the required insert, was digested with Bam H1 and Nar 1 and separated by electrophoresis on 1.2% agarose gel. A DNA band corresponding to a 519bp fragment was cut, eluted and purified. Following the same procedure, a 1300 bp DNA fragment was obtained by digestion of ptPBM-1 with Bam H1 and Nar 1. Equimolar amounts of 519 bp and 1300 bp DNA fragments were used for ligation following the standard procedure of Goodman, H.M., and MacDonald, R.J., Method. Enzymol 68, 75, (1979). The ligation mixture was extracted twice with a phenol:chloroform (1:1) mixture and the DNA was precipitated with two volumes of absolute ethanol. After dissolving the pellet in 50µl H₂O, the DNA was digested with Bam H1 under standard assay conditions and separated by electrophoresis on a 1% agarose gel. A DNA fragment containing 1819 bps, was cut, eluted from the gel and purified. This DNA fragment contained all the coding information required for the signal peptide

- 24 -

(20 amino acids) and the mature hybrid or tris-kringle PA molecule of 573 amino acids which corresponds to (UKaa¹⁻¹³¹-Ser-Glu-Gly-Asn-Ser-Asp)¹⁻⁹¹-t-PA depicted in figure 1(a).

The tris-kringle gene was then ligated to Bgl II cut expression vectors papilloma virus (BPV) which served as the complete expression vector. Conventional culture yielded the tris-kringle plasminogen activator of Figure 1(a).

Construction of Urokinase Kringle Sequence

In Examples 2 and 3, only the kringle part (a.a. 50-131) of urokinase is utilized and is inserted either before or after the double kringle region of t-PA. Fig. 6 depicts the construction of a nucleotide sequence, coding for a.a. 51-131 from the recombinant plasmid pUK 53. There is a convenient restriction site, Mst I, just after the nucleotide sequence corresponding to amino acid 131. However, none could be found around a.a. 50. Thus, a scheme for the creation of a restriction site around a.a. 50 (Nde I in this example) was formulated as shown in Fig. 8. The kringle region of urokinase corresponds to the nucleotide sequence from bp 284 (a.a 50) to bp 530 (a.a. 131).

About 10 µg of pUK 53 plasmid DNA was digested to completion with Sea I which cuts at bp 204 in the urokinase sequence. After phenol extraction and ethanol precipitation, the DNA pellet was dissolved in 50 µl of buffer solution (10 mM CaCl₂, 12 mM MgCl₂, 0.2 M NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA). To the reaction mixture was added 1 µl (2 units) of nuclease Bal 31 and the mixture was incubated at 30°C for 15 seconds (Legerski, R. J., J. L. Hodnett, and H. B. Gray, Jr., Nucleic Acid Res. 5, 145, 1978). The reaction was stopped by the addition of 5 µl of 0.4M EDTA. This reaction time was found to be sufficient to remove about 80 bp from each end of the DNA fragment. After phenol extraction and ethanol precipitation, the DNA was ligated to an oligonucleotide linker (10 bp) under standard reaction conditions. The oligomer linker

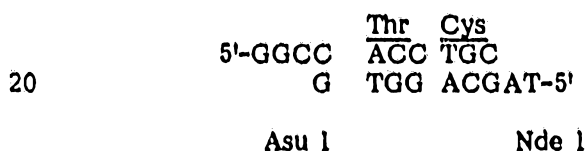
(Eco RI/Nde I linker) with the sequence, TGGAATTCCA, was designed to create an Nde I site (CATATG) when ligated to the DNA fragment end containing the sequence, TATG (corresponding to a.a. 51). In addition, the restriction site, Eco RI, was built into the linker to provide for subsequent cloning in a pUC 13
5 vector. After phenol extraction and ethanol precipitation, the DNA was digested with Eco RI and separated by electrophoresis on 1% preparative agarose gel. A DNA band corresponding to 340 bp, was cut, eluted and ethanol precipitated. About 40 ng of this DNA was ligated with about 0.4 µg of Eco RI cut pUC 13 vector DNA and used to transform competent *E. coli* JM 103 cells (Maniatis et
10 al., loc. cit. p. 250). About 1,000 recombinant colonies were obtained from 10 plates. The bacterial colonies were replica-plated on nitro-cellulose paper, and screened by in situ hybridization using a radioactive oligonucleotide probe (Grunstein et al. Proc. Nat'l. Acad. Sci USA 72, 3961, 1975). The oligonucleotide probe used was 18 bp long (TTCCATATGAGGGGAATG) and contains the first
15 five nucleotides from the Eco RI/Nde I linker and the next 13 bases from the urokinase sequence corresponding to a.a 51 to 54. About 12 clones showed a moderate to strong signal on X-ray film. Miniplasmid DNA prepared from these 12 clones was digested with Nde I and separated by electrophoresis on 1% agarose gel. One clone, pUKKnd 16, was found to contain the newly generated
20 Nde I site. This plasmid DNA, after digestion with Nde I and Mst I, was separated by electrophoresis on 1.4% agarose gel to obtain a 246 bp DNA fragment. This DNA fragment contains the urokinase nucleotide sequence coding for a.a. 51 to 131. The DNA sequence for the missing a.a. 50 (Cys) is incorporated into the oligomer linker as shown in Fig. 7 and 8.

Example 291-(UKaa⁵⁰⁻¹³¹-Ser-Glu-Gly-Asn-Ser-Asp)-92-t-PA

The urokinase kringle sequence as shown in Fig. 1(b) was inserted before the double kringle region of t-PA i.e. between a.a. 91 and 92. As described in the preceding paragraph, Nde I and Mst I digestion of pUKKND 16 plasmid DNA gave rise to a 246 bp sequence which corresponds to a.a. 51 to 131 of urokinase. The two ends of the UK kringle sequence (246 bp) were inserted into the t-PA gene between nucleotide no. 462 (a.a. 91) and 463 (a.a. 92) through the use of two oligonucleotide linkers. The procedure followed is shown in Fig. 7.

About 50 µg of ptPBM-1 plasmid DNA was digested with Eco RI and a 740 bp DNA fragment was isolated by electrophoresis in 1% agarose gel. This DNA was then digested partially with Sau 961 (isoschizomer of Asu I) for the isolation and purification of a 385 bp DNA fragment.

Two complementary oligonucleotide sequences were synthesized by the phosphotriester method (Crea *et al.*, Proc. Nat'l. Acad. Sci (USA) **75**, 5765, 1978) which code for amino acid 91 (Thr) of t-PA and amino acid 50 (Cys) of the urokinase kringle.



The oligonucleotide linker is flanked by Asu I and Nde I restriction sites. Only the upper oligonucleotide (GGCCACCTGC) was phosphorylated at its 5' end.

About 1 µg of the 385 bp DNA fragment was ligated overnight with approximately 1 µg of oligomer linker. After phenol extraction and ethanol precipitation, the DNA was digested for 2 hours with Eco RI and separated by electrophoresis on 1.2% agarose gel to obtain a 394 bp DNA fragment. This DNA

- 27 -

fragment contains the nucleotide sequences coding for the signal peptide (35 a.a.) and N-terminal peptide a.a. 1 - 91 of t-PA. In addition, it also restores the DNA sequence for a.a. 50 (Cys) of the UK kringle not present in its 246 bp DNA fragment (Fig. 6).

5 In order to obtain the C-terminal t-PA sequence, amino acids 92 onward, about 50 µg of ptPBM-1 plasmid DNA was digested with Ava II and a 747 bp DNA fragment was isolated from 1% agarose gel. Two complementary DNA fragments of 27 and 30 bp long were synthesized by the phosphotriester method. As shown in Fig. 7, this DNA linker codes for the hexapeptide Ser-Glu-Gly-Asn-
10 Ser-Asp and also restores the missing a.a. 92 to 94 (Cys-Tyr-Glu) in the Ava II cut 747 bp DNA. Only the lower oligomer (30 mer) was phosphorylated at its 5' end. About 1 µg of 747 bp DNA and 1 µg of oligomer linker were ligated overnight at 15°C. After phenol extraction and ethanol precipitation, the DNA was digested for 2 hours with Eco RI to obtain a 354 bp DNA fragment from
15 1.4% agarose gel. About 500 ng of each of the three DNA fragments, 394 bp, 246 bp (UK Kringle) and 354 bp, were ligated overnight using T4 DNA ligase. After phenol extraction and ethanol precipitation, the DNA was digested with Eco RI and a 994 bp DNA fragment was isolated and purified from the 1% agarose gel. This DNA, after ligation with an equimolar amount of Eco RI cut
20 pUC 13 vector, was used to transform competent E. coli JM 103 cells. Miniplasmid DNA preparations from 12 recombinant clones were digested with Eco RI. One clone containing the required insert of 994 bp, was grown in 1 liter LB medium for large scale preparation of plasmid DNA. About 10 µg of this plasmid DNA was digested with Bam HI and Nar I and a DNA fragment
25 corresponding to 700 bp size was purified from the 1% agarose gel.

The 3' end of the t-PA gene was obtained by digestion of about 10 µg of ptPBM-1 plasmid DNA with Bam HI and Nar I. After separating the reaction

- 28 -

mixture by electrophoresis on 1% agarose gel, an approximate 1300 bp DNA fragment was isolated by electroelution and purified.

Approximately equimolar amounts of the two DNA fragments of 700 bp and 1300 bp size, were ligated overnight and a 2000 bp DNA fragment was recovered from 1% agarose gel. This DNA, flanked by the restriction enzyme Bam HI sequence, was inserted at the Bgl II site of the BPV expression vector. This DNA codes for a protein containing a total of 650 amino acids - 35 amino acids for the signal peptide and 615 amino acids for the mature protein. Conventional culture, recovery, isolation and purification techniques yielded the tris-kringle plasminogen activator of Fig. 1(b).

Example 3

261-(Ser-Glu-Gly-Asn-Ser-Asp-UKaa⁵⁰⁻¹³¹)-262-t-PA

In this example, the urokinase kringle sequence is inserted just after the double kringle region of the t-PA gene i.e. between the sequences corresponding to a.a. 261 (Cys.) and 262 (Ser). Fig. 8 shows the scheme detailing the various steps involved in the production of this tris-kringle PA.

About 10 µg of pWP 42 plasmid DNA was digested with Hga I and a 400 bp DNA fragment was isolated from 1% agarose gel. This DNA fragment contains the sequence corresponding to part of the kringle region of t-PA, i.e., amino acids 135-261. It should be noted that the double kringle region of t-PA ranges from amino acids 92 to 261, with a hexapeptide (amino acids 174-179) joining the two kringles. An oligonucleotide linker consisting of two DNA sequences - 24 mer and 21 mer as depicted in Fig. 8 - was synthesized using the phosphotriester method. This oligomer linker codes for the hexapeptide linker as well as for missing amino acid 50 (Cys) of the UK kringle and is flanked by restriction enzyme sequences for Hga I and Nde I. Only the upper oligo-

- 29 -

nucleotide, 23 mer was phosphorylated at its 5' end and it was ligated to the Hga 1 end of the 400 bp DNA fragment from t-PA. The 421 bp DNA product was isolated from preparative 1% agarose gel.

5 The post kringle part of t-PA was obtained by digestion of 10 µg of pWP 42 plasmid DNA with Rsa 1 followed by isolation of 501 bp DNA from 1.2% agarose gel. This DNA represents a.a. 269 to 435 of the t-PA molecule. Two complementary oligonucleotide sequences of 22 bases as depicted in Fig. 8 were synthesized by the phosphotriester method. This DNA linker, when ligated to the 501 bp DNA at its 5' end restores the missing amino acids from 262 to 268.

10 Approximately 1 µg of the 501 bp DNA and 1 µg of the linker DNA were ligated overnight and a DNA band corresponding to 523 bp size was purified from 1% agarose gel.

The three DNA fragments of sizes 421 bp, 246 bp (UK Kringle) and 523 bp, were ligated in approximately equimolar amounts at 15°C for 16 hours. 15 After phenol extraction and ethanol precipitation, the DNA was cut with Eco R1 and a DNA fragment of 741 bp was isolated. This DNA, flanked by two Eco R1 restriction sites, was amplified in pUC 13 vector system as described above.

10 µg of ptPBM-1 plasmid DNA in which the Eco R1 site in multiple cloning site had been removed, was digested to completion with Eco R1 and the 20 larger vector DNA fragment of about 4.0 Kb was isolated from 1% agarose gel. About equimolar amounts of Eco R1 cut vector DNA and the 741 bp DNA were ligated and the product used to transform competent E. coli JM 103 cells. Miniplasmid DNA, prepared from 12 recombinant clones, was digested with Bam H1 to look for the desired insert of about 1.8 Kb. The correct orientation of the 25 741 bp DNA in the insert was determined by digestion of the plasmid DNA with Nar 1 and Mst 1. One clone, ptPUHYC, when digested with Nar 1 and Mst 1 was found to contain a fragment of approximately 700 bp in correct orientation.

- 30 -

The 1.8 Kb DNA, obtained by digestion of ptPUHYC plasmid DNA with Bam H1, contains the nucleotide sequence coding for a protein of 650 amino acids - 35 amino acids for the signal peptide and 615 amino acids for mature protein corresponding to the product of Fig. 1(c).

5 The tris-kringle gene is then ligated to Bgl II cut bovine papilloma virus (BPV) which serves as the complete expression vector. Conventional culture yields the tris-kringle plasminogen activator of Fig. 1(c).

TETRA-KRINGLE PLASMINOGEN ACTIVATOR

Prothrombin cDNA:

10 A cDNA clone for the prothrombin gene was isolated from the human liver cDNA library following the procedure of Friezner et al., Biochemistry, 22, 2087, 1983. The clone, pPTR, contains complete coding information for the mature protein of 579 amino acids. The double kringle region of prothrombin extends from amino acid 65 (bp 319) to amino acid 248 (bp 840), a 184 amino acid
15 peptide. Each of the two kringles, PTK1 (amino acids 65-143) and PTK 2 (amino acids 170-248) are 79 amino acids long and are joined by a peptide of 26 amino acid length (amino acids 144-169).

Preparation of Prothrombin Double Kringle Sequence

20 The DNA sequence representing the double kringle region was isolated from the prothrombin cDNA in two steps (Fig. 12).

 In the first step, 10 µg of pPTR plasmid DNA was digested with Ava I and a 706 bp DNA was isolated from 1% agarose gel. This DNA was treated with Bal 31 for 7.5 seconds to remove about 38 bp from either end of the DNA (Legerski et al., Nucleic Acid Res., 5, 145, 1978). After phenol extraction and
25 ethanol precipitation, the DNA was ligated to an Eco RI linker, GGAATTCC, at

4°C. for 15 hours. This linker, when ligated to DNA ending with sequences CTGAG or TGAG (amino acid 67, Glu) will produce a new restriction sequence for Mst II (CCTGAGG) at the N-terminal of the double kringle (amino acid 67). After cutting with Eco R1, a DNA fragment of 630 bp size was isolated. This
5 DNA, after ligation in equimolar amounts with Eco R1/pUC 13, was then used to transform competent *E. coli* JM 103 cells. Initial screening for the desired clone was performed by in situ hybridization with a ³²P labeled probe, GAATTCCTGAGGGTCTG, containing nucleotide sequences for amino acids 66 to 68. Twelve clones, exhibiting a strong signal on X-ray film, were grown for a
10 miniplasmid preparation. After digestion of plasmid DNA with Mst II and Bam H1, one clone, pPTK-5' was found to contain the required insert of about 630 bp and also the newly created Mst II site at 322 bp.

The second step involved the editing of the 3' end of the kringle region, around amino acid 248, using a similar approach as described above.
15 About 10 µg of pPTK-5' plasmid DNA was digested to completion with Bam H1. The DNA was then treated with Bal 31 for 15 seconds at 30°C. to remove about 82 bp from both ends of the DNA. After phenol extraction and ethanol precipitation, the DNA was ligated to a Eco R1/Fsp 1 linker, CGCAGAATTCTGCG. As the name suggests, the linker generates an Fsp I
20 sequence (TGCGCA) at any DNA sequence ending in TG-. After cutting thoroughly with Eco R1, a 546 bp DNA was isolated from 1.2% agarose gel and then ligated to Eco R1 cut pUC 13. The recombinant plasmid was then used to transform competent *E. coli* JM 103 cells. About 1000 recombinant clones were screened in situ using a ³²P-labelled oligomer with the sequence
25 CTCAACTATTGCGCAGAA (amino acids 245 to 248). Plasmid DNA prepared from 12 potential clones, was cut with Mst II and Fsp 1 and run on a 1% agarose gel. One clone, pPT2K, was found to contain the required insert of 546 bp size.

- 32 -

This DNA codes for a total of 182 amino acids, amino acids 67-248, of the double kringle region. The nucleotide sequence for the remaining two amino acids, at positions 65 (Cys) and 66 (Ala), are added via the oligomer linker as shown in Fig. 13.

5

Example 4

91(PTK_{aa}⁶⁵⁻²⁴⁸-Ser-Glu-Gly-Asn-Ser-Asp)-92-t-PA

The double kringle sequence of prothrombin, amino acids 65-248, was inserted before the double kringle region of t-PA (i.e. between amino acids 91 and 92) to give rise to tetra kringle-PA. As described in the preceding paragraph, Mst II + FspI or Eco RI digestion of pPT2K plasmid DNA gives rise to a 546 bp DNA fragment which codes for amino acids 67 to 248 of prothrombin. Through the use of two oligonucleotide linkers, the two ends of the 546 bp DNA were inserted into the t-PA gene between nucleotide 462 (amino acid 91) and 463 (amino acid 92). The scheme followed is shown in Fig. 13.

15

The 354 bp DNA, containing the nucleotide sequence for the peptide linker-Ser-Glu-Gly-Asn-Ser-Asp as well as for the amino acid 92 to 204 of t-PA, was prepared as described in Example 2 and Fig. 7. Equimolar amounts of the 354 bp DNA and 546 bp DNA (prothrombin kringles) was ligated at 4°C. for 16 hours. The DNA was cut with Eco RI and a 900 bp DNA fragment was isolated from agarose gel. This DNA after ligation to Eco RI cut pUK 13 vector was used to transform E. coli JM 103 cells. Miniplasmid preparation obtained from 12 recombinant clones were digested with Eco RI and Mst II. One clone, pPT2KTPK, was found to contain the required insert of 900 bp. This DNA codes for a total of 300 amino acids, 182 amino acids for prothrombin (amino acids 67-248), a hexapeptide sequence and 112 amino acids for t-PA (amino acids 92-204).

25

The preparation of 385 bp DNA obtained by digestion of ptPBM-1 plasmid with Eco R1 and Asu 1 is described in Fig. 7. Two complementary oligonucleotide sequences of 12 bp each, were synthesized by the phosphotriester method. This linker restores the missing amino acids, Cys and Ala, of the prothrombin kringle region and is flanked by the Asu 1 and Mst II recognition sequences. About 500 ng of DNA (385 bp) was ligated with 1 µg of phosphorylated linker as shown in Fig. 13. After cutting with Eco R1, a 402 bp DNA fragment was isolated. About equimolar amounts of 402 bp DNA and 900 bp DNA were ligated, cut with Eco R1 and a 1302 bp DNA fragment was isolated from the 1% agarose gel. This DNA was subcloned in Eco R1 cut pUC 13 vector. Plasmid DNA, isolated from 12 recombinant clones, was digested with Eco R1. One clone, pNPT2KPAK, containing the required insert, was grown for large scale preparation of plasmid DNA. The plasmid DNA was digested to completion with Bam H1 and then partially with Nar 1 to obtain a 986 bp DNA fragment. Similarly, a 1300 bp DNA fragment was obtained from digestion of ptPBM-1 with Bam H1 and Nar 1. The two DNA fragments, 986 bp and 1300 bp, in equimolar amounts were ligated, cut with Bam H1 and a 2286 bp DNA fragment was isolated from 1% agarose gel electrophoresis. The DNA, flanked by the Bam H1 sequences, was inserted at the Bgl II site of the BPV expression vector. This DNA codes for a total of 752 amino acids - 35 amino acids for the signal peptide and 717 amino acids for the mature protein. The mature protein, with an estimated molecular weight of about 92,000 contains 184 amino acids from the prothrombin double kringle region, a hexapeptide linker and complete t-PA sequence of 527 amino acids.

Expression and Biochemical Characterization of

Hybrid Plasminogen Activators

Two of the hybrid plasminogen activators (h-PA) shown in Fig. 1(a) and Fig. 1(b) were prepared for expression in the BPV-I based expression vector system. These h-PA's are throughout this application referred to as Hybrid A and Hybrid B, respectively.

The complete gene sequences, flanked by BamHI for Hybrid A (1.8 Kb, Fig. 5) and Hybrid B (2.0 Kb, Fig. 7) were inserted into the Bgl II cut plasmid p341-3 (for details see Methods and Materials). Miniplasmid preparations from 12 recombinant clones were prepared and digested individually with Nar I, Bgl II or BamHI and Ava I. This was used to confirm the presence of hybrid genes as well as to determine the orientation of the insert. Only one orientation of the gene, i.e., in the direction of the metallothionein promoter, is desirable, because it places the expression of the gene under the control of that promoter. In addition, the SV40 poly-A sequence located just behind the gene, would process the RNA transcript of the gene by polyadenylation at its 3'-end for efficient translation of the gene. Two recombinant clones pHyb AMT-43 (for Hybrid A) and pHyb BMT-50 (for Hybrid B) were obtained.

About 1 µg of the plasmid DNA obtained from the above mentioned clones, was digested with BamHI and then dephosphorylated with bacterial alkaline phosphatase. To this was inserted a complete 8.0 Kb BamHI cut BPV-I genome. Two expression plasmids pHyb-AMTBPV-438 and pHyb-BMTBPV-504 (simply referred to as p438 and p504) containing the genes coding for Hybrid A and Hybrid B, respectively, were obtained. A complete map showing the relative positions of various components of the expression plasmids is shown in Fig. 14.

The two expression plasmids containing the genes encoding for hybrid t-PA/urokinase molecules were transfected into mouse C127 cells by the

calcium phosphate precipitation method (Graham et al., Virology, 52, 456 (1973)). Foci of morphologically transformed cells were subcultured and screened for gene expression. Initial screening for fibrinolytic activity in the medium was done on a fibrin-agar plate as shown in Fig. 15(a) (Ploug et al. Biochim. Biophys. Acta, 24, 278 (1957)). From each transfection, an average of 50% of foci transformed with p504 (Hybrid B) and 5% of foci transformed with p438 (Hybrid A) showed positive fibrinolytic activity in the culture medium. Several high producers were selected and cell lines were expanded from individual foci. The two cell lines with which most of the preliminary biochemical and immunological characterizations of the gene products were performed were labeled clone 5A5 for Hybrid B and clone 16C1 for Hybrid A.

Biochemical Characterization

The enzymatic activities of the hybrid molecules were assessed by fibrin-agar assay and amidolytic activity assay using synthetic substrates S-2444 and S-2251 (Shimoda et al., Thromb. Haemostas., 46, 507 (1981)). Approximately 1-5 units/ μ l active enzyme were secreted into the medium in 16 to 18 hours. Natural t-PA and urokinase are synthesized as precursors and secreted from the cells after the signal sequences are cleaved off to become the mature protein. Furthermore, both t-PA and urokinase are glycosylated. To determine whether the h-PA's secreted by the transfected mouse cells were processed in a similar fashion as natural t-PA and urokinase, the hybrid molecules were analyzed by SDS/polyacrylamide gel (PAGE) electrophoresis followed by fibrin-agar overlay (Granelli-Piperno et al., J. Exp. Med., 148, 223, (1978)). As shown in Fig. 15(b), the active enzyme from Hybrid B-containing medium has a molecular weight of 76,000 and Hybrid A, 71,000. These are in good agreement with the molecular weight calculated from the inserted gene.

- 36 -

Hybrid B was purified from harvest medium in a manner similar to that used for t-PA purification. Amino acid sequence analysis indicated that the N-terminal of Hybrid B is correctly processed, having the sequence identical to the N-terminal region of mature t-PA: Ser-Tyr-Gln- ; in addition, an N-terminal
5 sequence, Ile-Lys-Gly-, corresponding to the amino acid at the activation cleavage site Arg-Ile was present. It is concluded that Hybrid B purified from the harvest medium existed mainly as an activated, two chain form. By addition of a protease inhibitor (Aprotinin) to the harvest medium, a single chain h-PA molecule is obtained.

10 Immunoprecipitation followed by SDS/PAGE of ³⁵S-labeled harvest media showed that, under non-reducing conditions, bands at the position corresponding to an apparent molecular weight of 71,000-76,000 were observed in the sample from cell line 5A5 (Hybrid B) and 16C1 (Hybrid A), respectively, but not in the control sample. The fibrinolytic activity of the culture media from 5A5
15 cells as well as purified t-PA (American Diagnostics, Inc., Greenwich, CT) was neutralized by anti-t-PA antiserum, but not by anti-urokinase antiserum, suggesting that although the Hybrid B contains a urokinase kringle in addition to t-PA, the protease domain of Hybrid B was recognized and neutralized by anti-t-PA. Anti-urokinase antibody may bind to the urokinase kringle portion of the
20 hybrid molecule, but this binding, if any, does not interfere with the proteolytic activity conferred by the protease domain at the C-terminus of the hybrid molecule.

The poly-kringle plasminogen activators of this invention are used in treatment of vascular accidents in mammals in the same manner and through the
25 same delivery vehicles as t-PA itself. Thus the poly-kringle plasminogen activators of this invention may be formulated into pharmaceutical compositions

by dissolving or suspending the polypeptides in suitable pharmaceutically acceptable vehicles such as those known to the art as applied to t-PA. Administration to a mammal in need thereof by intravascular injection or infusion is conducted following techniques already established with t-PA itself. An intravenous primary dose of about 440 IU/kg body weight is normal, followed by continuing infusion of about 440 IU/kg/hr for about 6 to 12 hours is conventional practice when using t-PA.

Hybrid B exhibits properties very similar to those of t-PA in the following assays: fibrin clot binding; amidolytic activity toward smaller substrates (S-2288-D-Ile-Pro-Arg-p-nitroanilide) and fibrin-dependent plasminogen activation. In the rabbit and dog, Hybrid B can be detected up to 120 minutes after injection compared with 2 to 5 minutes for t-PA. The animals seem to exhibit much less systemic bleeding with Hybrid B than is seen with t-PA. As a result, fibrinolysis is sustained over a longer period of time and reocclusion is avoided.

Also provided is a process for converting plasminogen into plasmin which comprises contacting said plasminogen with a polypeptide according to this invention. In a further aspect this invention provides a process for disrupting blood clots in a mammal which comprises administering into the vascular system of said mammal a polypeptide according to this invention.



-38-

The claims defining the invention are as follows:

1. A hybrid plasminogen activator containing plural polypeptide kringles, at least one of said kringles being heterologous and at least one being homologous; adjacent homologous and heterologous kringles being separated by an amino acid linker sequence.
2. A plasminogen activator according to Claim 1 which contains from 2 to 6 kringles.
3. A tetra-kringle plasminogen activator according to Claim 1.
4. A tris-kringle plasminogen activator according to Claim 1.
5. A plasminogen activator according to any one of Claims 1 to 4 containing a polypeptide sequence corresponding to the amino acid sequence 92 to 261 of t-PA.
6. A plasminogen activator according to any one of Claims 1 to 4 containing a polypeptide sequence corresponding to one of the following amino acid sequences: 1 to 261 of t-PA,
92 to 527 of t-PA,
1 to 131 of urokinase,
50 to 131 of urokinase, or 65 to 240 of prothrombin.

.....
.....
.....
.....



- 39 -

7. A plasminogen activator according to any one of claims 1 to 6 in which the kringles or at least one pair of kringles is connected by a linker sequence containing 6 to 20 natural amino acids.

8. A plasminogen activator as claimed in Claim 7 in which the linker contains 6 to 10 natural amino acids.

9. A plasminogen activator as claimed in Claim 8 in which the linker is L-Ser-L-Glu-Gly-L-Asn-L-Ser-L-Asp or L-Thr-L-Asp-L-Ala-L-Glu-L-Thr-L-Glu.

10. A plasminogen activator having a formula selected from one of the following:

91(PTKaa⁶⁵⁻²⁴⁸-Ser-Glu-Gly-Asn-Ser-Asp)-92-t-PA, as hereinbefore defined,

(UKaa¹⁻¹³¹-kringle linker)¹⁻¹⁹¹ t-PA, as hereinbefore defined,

and

91-(UKaa⁵⁰⁻¹³¹-kringle linker)-92t-PA as hereinbefore defined; in which the kringle linker is a polypeptide moiety containing 6 to 10 natural amino acids.

11. A plasminogen activator of formula 261-(kringlelinker-UKaa⁵⁰⁻¹³¹)-262-t-PA as hereinbefore defined in which the kringle linker is a polypeptide moiety containing 6 to 20 natural amino acids.

12. A plasminogen activator according to Claim 10 or Claim 11 in which the kringle linker is L-Ser-L-Glu-Gly-L-Asn-L-Ser-L-Asp.

- 40 -

13. A DNA unit having a nucleotide sequence encoding for the polypeptide of any one of Claims 1 to 12.

14. A cloning vector that includes the DNA unit of Claim 13.

15. A plasmid selected from pHyb-AMTBPV-438 or pHyb-BMTBPV-504 as hereinbefore defined.

16. A microorganism or cell culture transformed with a vector according to Claim 14 or Claim 15.

17. A process for preparing a plasminogen activator according to any one of Claims 1 to 12 which comprises constructing by recombinant DNA techniques, a replicable expression vector containing the DNA sequence encoding for said hybrid plasminogen activator, transforming suitable host cells to introduce said expression vector and obtain transformed host cells, culturing said transformants to express said hybrid plasminogen activator and recovering said hybrid plasminogen activator.

18. A pharmaceutical composition comprising a plasminogen activator according to any one of Claims 1 to 12 and a pharmaceutically acceptable carrier.

19. A plasminogen activator as claimed in claim 1 substantially as hereinbefore described with reference to any one of the drawings.

DATED: 15th December, 1989

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Attorneys for:

AMERICAN HOME PRODUCTS CORPORATION



61102/86

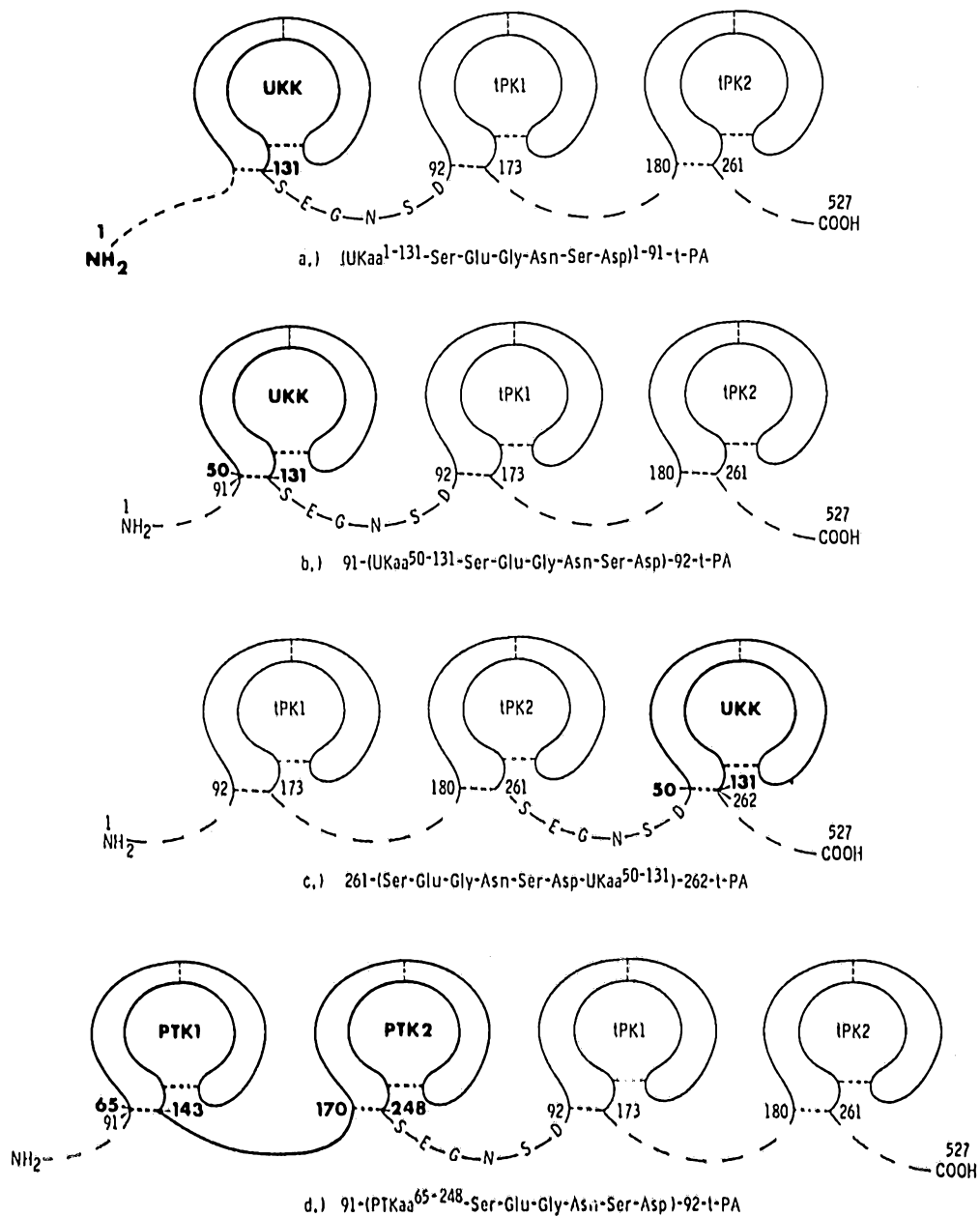


FIG. 1

20179 94.11.9

RESTRICTION OF MAP OF tPA CLONE pWP42 FROM Wi-38

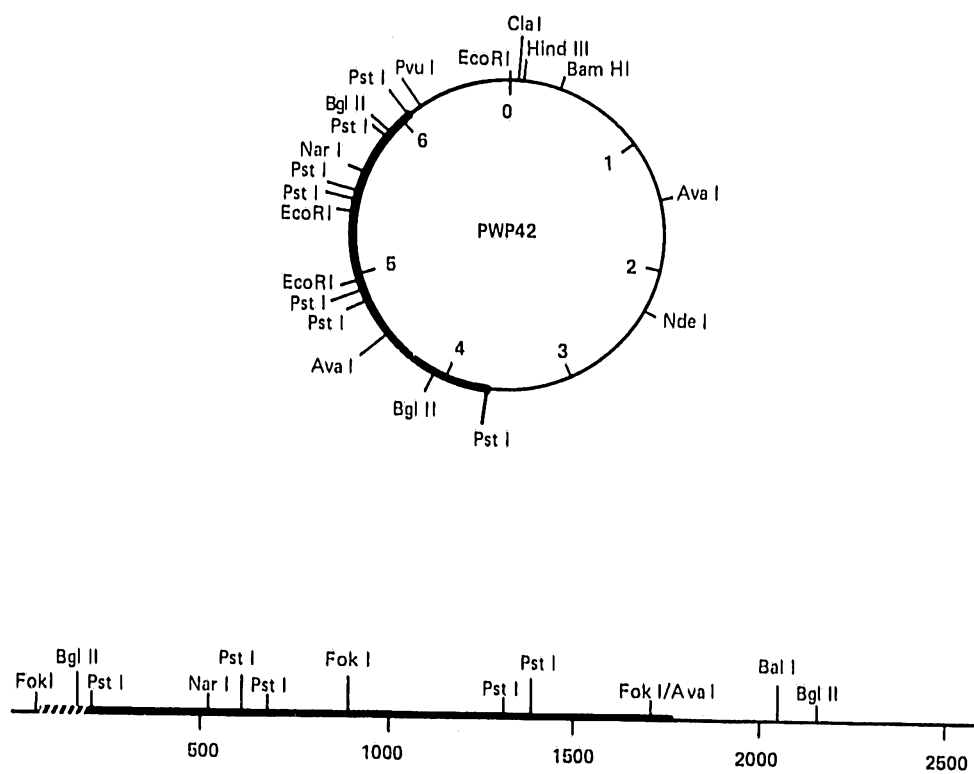


FIG. 2

001793X-1-9

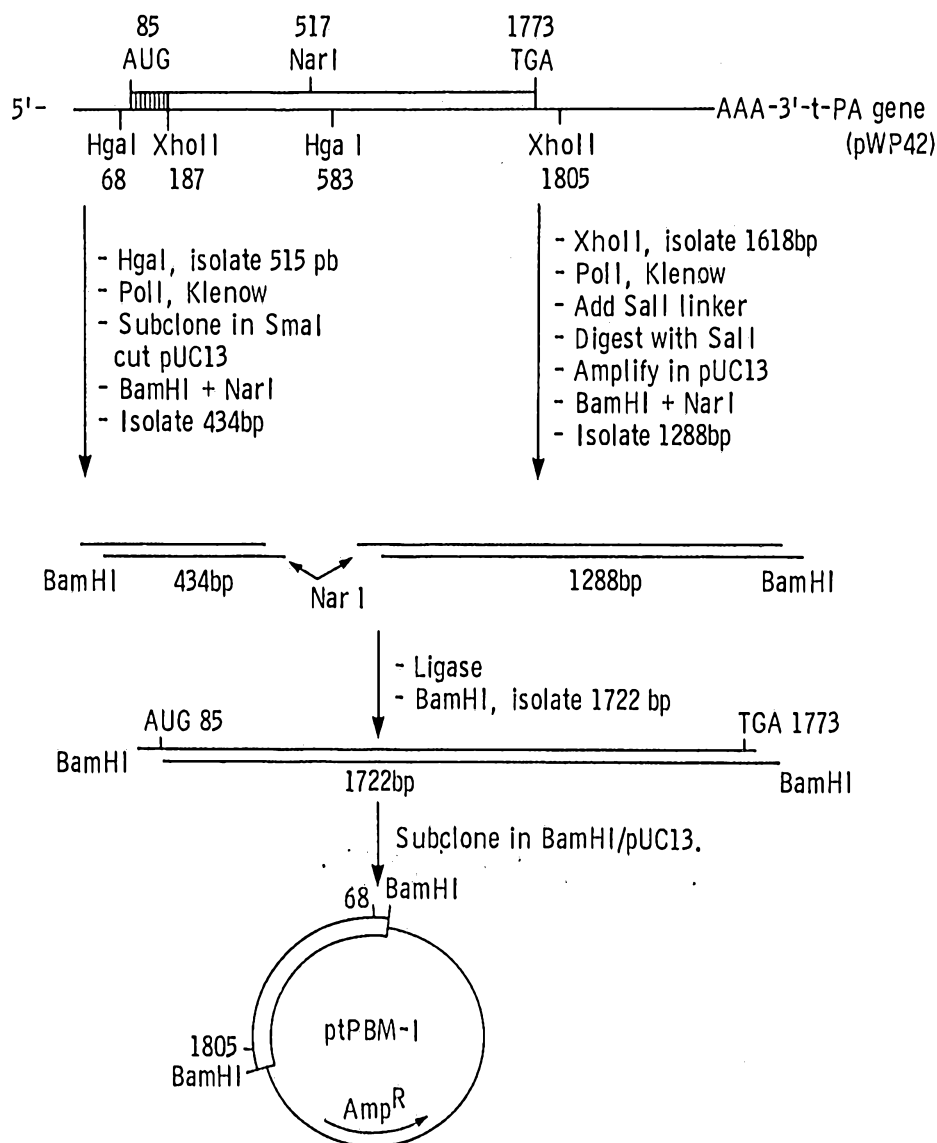


FIG. 3

00119 3x 119

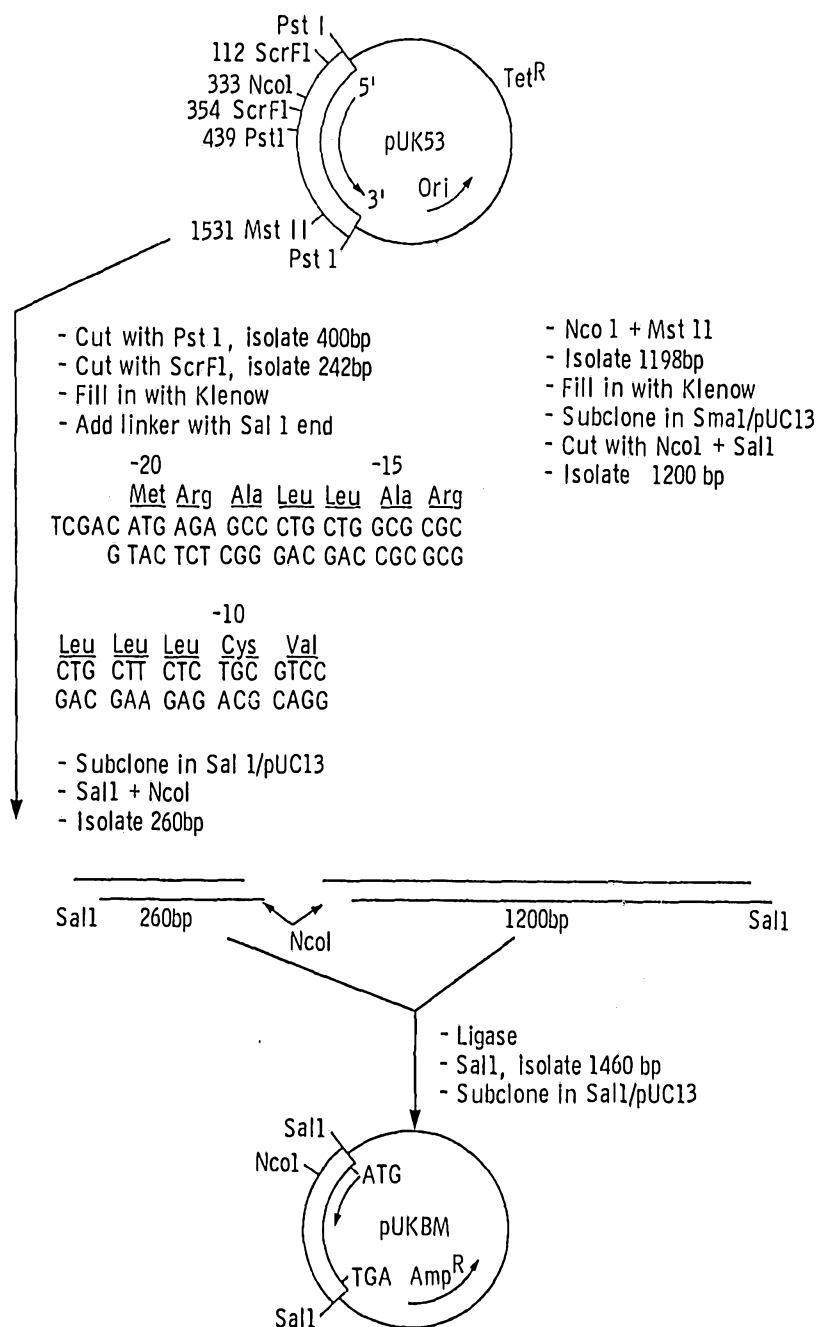


FIG. 4

50119 94.11.9

PREPARATION OF TRIS-KRINGLE PA OF FIGURE (1a)

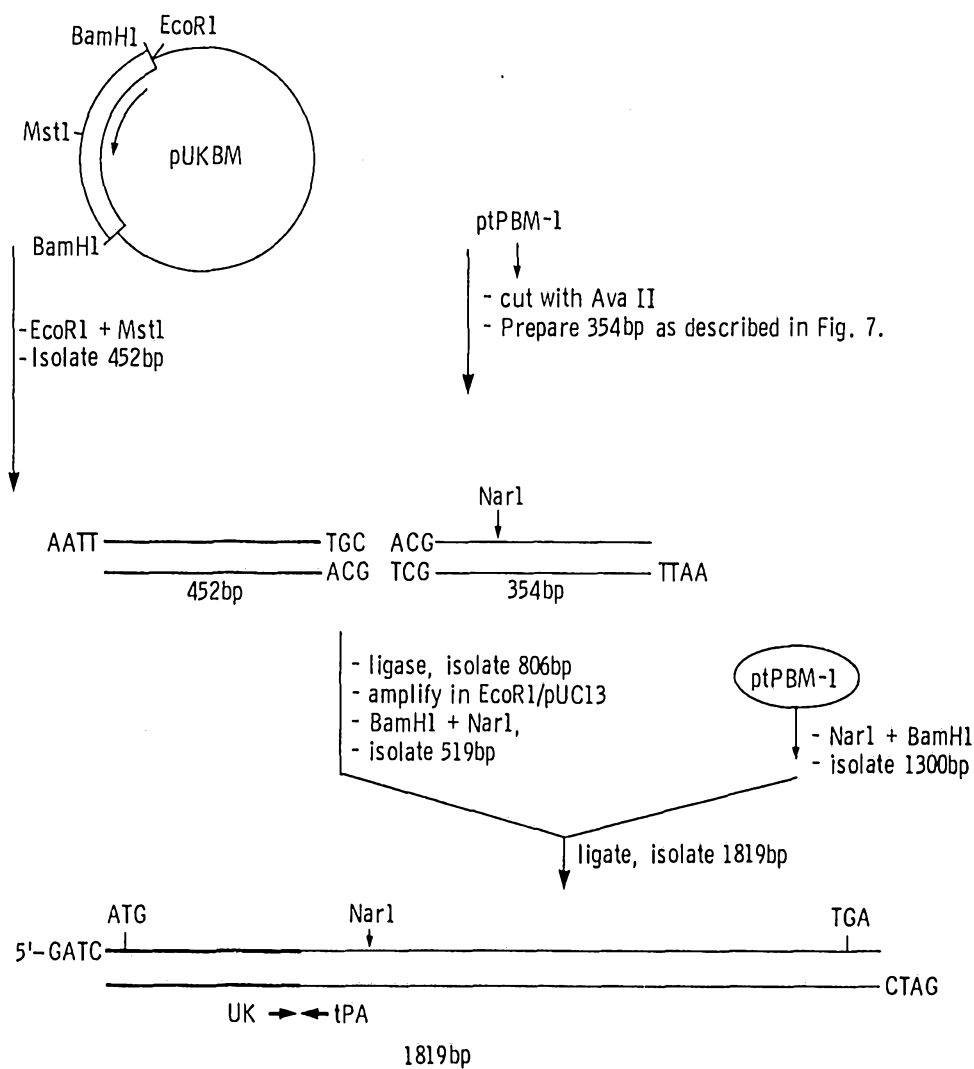


FIG. 5

5' 3' 5' 3' 5' 3' 5' 3'

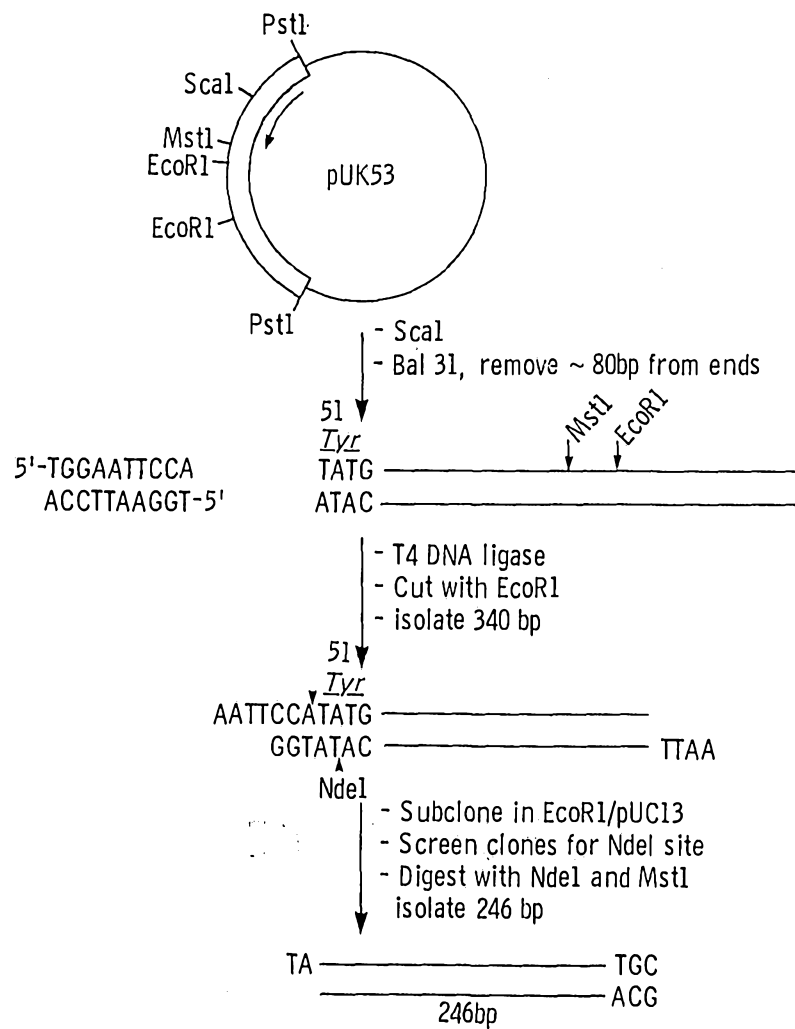


FIG. 6

001 10 00 110



PREPARATION OF TRIS-KRINGLE PA OF FIG. 1(c)

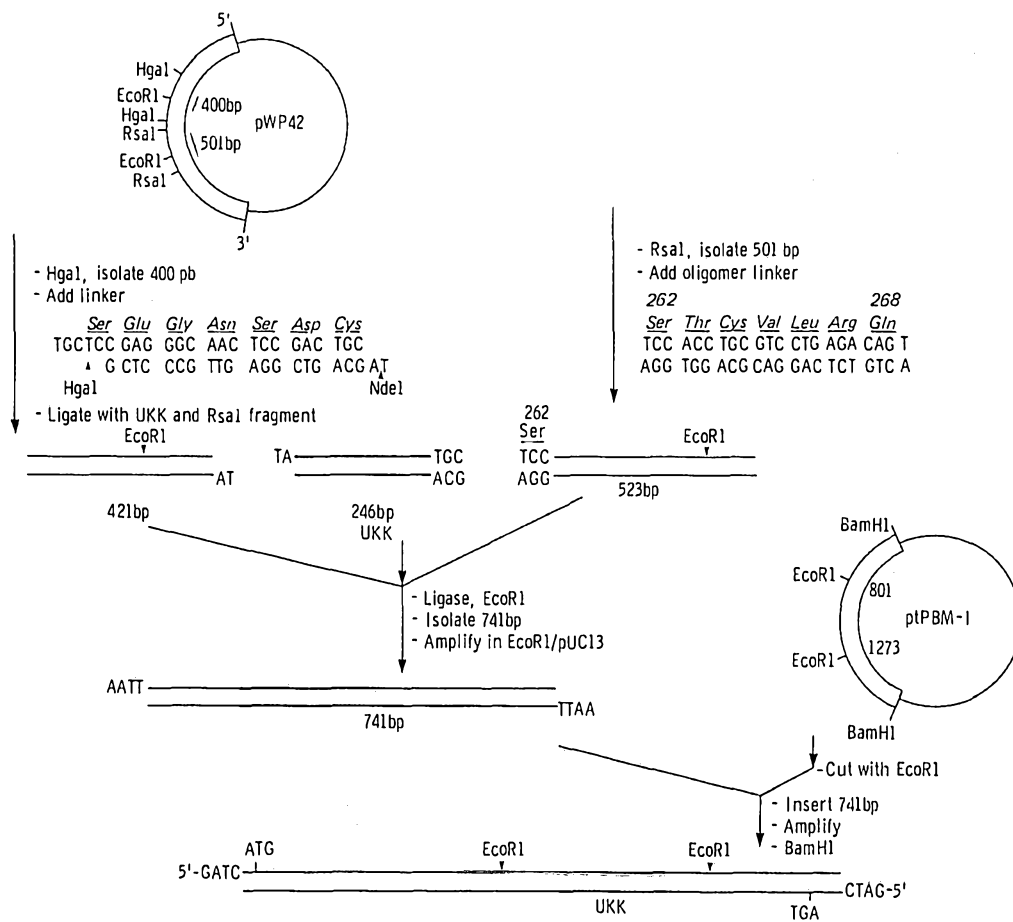


FIG. 8

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61102/86

TRIS-KRINGLE PA OF FIG. (1a).

1	11	21	31	41	51	
GGATCCTCGACATGAGAGCCCTGCTGGCGCGGCTGCTTCTCTGGTCTGGTGGTGGAGCG						UK
MetArgAlaLeuLeuAlaArgLeuLeuLeuCysValLeuValValSerAsp						signal peptide
61	71	81	91	101	111	20 a.a.
ACTCCAAAGGCAGCAATGAACCTTCATCAAGTTCCATCGAAGTGTGACTGTCTAATGGAG						
SerLysGlySerAsnGluLeuHisGlnValProSerAsnCysAspCysLeuAsnGlyGly						
121	131	141	151	161	171	
GAACATGTGTGTCCAAACAGTACTTCTCCACACATTCAGTGGTGCACACTGCCCAAGAAAT						UK Sequence
ThrCysValSerAsnLysTyrPheSerAsnIleHisTrpCysAsnCysProLysLysPhe						(aa1-131)
181	191	201	211	221	231	
TCCGAGGGCAGCACTGTGAATAGATAAGTCAAAHACCTGCTATGAGGGGAAATGGTCACT						
GlyGlyGlnHisCysGluIleAspLysSerLysThrCysTyrGluGlyAsnGlyHisPhe						
241	251	261	271	281	291	
TTTACCGAGGAAGGGCAGCACTGACACCAATGGGCGGGCCCTGCTGCCCTGGAACTCTG						
TyrArgGlyLysAlaSerThrAspThrMetGlyArgProCysLeuProTrpAsnSerAla						
301	311	321	331	341	351	
CCACTGTCTTCAGCAACGTAACATGCCACAGATCTGATGCTCTTCAGCTGGGCGCTGG						
ThrValLeuGlnGlnThrTyrHisAlaHisArgSerAspAlaLeuGlnLeuGlyLeuGly						
361	371	381	391	401	411	
GGAAACATAATTACTGCAGSACCCAGACACCCGGAGGGCAGCCCTGGTGTATGTGCAGG						
LysHisAsnTyrCysArgAsnProAspAsnArgArgArgProTrpCysTyrValGlnVal						
421	431	441	451	461	471	
TGGGCTTAAGCCGCTTGTCCAGAGTGCATGGTGCATGACTGCAGCGAGGGCACTCCG						Hexapeptide
GlyLeuLysProLeuValGlnGluCysMetValHisAspCysSerGluGlyAsnSerAsp						Linker
481	491	501	511	521	531	
ACTGCTACGAGGACCGGGGCTCAGCTACAGGGGCGAGTGGAGCACAGCGGAGAGTGGCG						
CysTyrGluAspGlnGlyIleSerTyrArgGlyThrTrpSerThrAlaGluSerGlyAla						
541	551	561	571	581	591	
CCGAGTGCACCACTGGACACGAGCGCGTTGGCCAGAGCCCTACAGCGGGCGGAGGC						
GluCysThrAsnTrpAsnSerSerAlaLeuAlaGlnLysProTyrSerGlyArgArgPro						
601	611	621	631	641	651	
CAGACGCCATCAGGCTGGGCTGGGGACCCACCACTACTGCAGAAACCCAGATCGAGACT						
AspAlaIleArgLeuGlyLeuGlyAsnHisAsnTyrCysArgAsnProAspArgAspSer						
661	671	681	691	701	711	
CAAGGCCCTGGTGTACGTCTTTAGGGGGGAGTACAGCTCAGAGTTCTGCAGCACCC						
LysProTrpCysTyrValPheLysAlaGlyLysTyrSerSerGluPheCysSerThrPro						
721	731	741	751	761	771	
CTGCCTGCTGTAGGGAAACAGTGAAGTCTACTTTGGGAATGGGTACGCCATACCGTGGCA						
AlaCysSerGluGlyAsnSerAspCysTyrPheGlyAsnGlySerAlaTyrArgGlyThr						
781	791	801	811	821	831	
CGCACAGCCTCACCGAGTGGGCTGCCTCCTGCCCTCCCGTGGATTCCATGATCCTGATAG						
HisSerLeuThrGluSerGlyAlaSerCysLeuProTrpAsnSerMetIleLeuIleGly						
841	851	861	871	881	891	
GCAAGGTTTACACAGCACACACCCAGTGCACAGCCACTGGGCTGGGCAACATAATT						
LysValTyrThrAlaGlnAsnProSerAlaGlnAlaLeuGlyLeuGlyLysHisAsnTyr						

FIG. 9

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Example A (continued)

901 911 921 931 941 951
ACTGCCGGGAATCCTGATGCGGATGCCAAGCCCTGGTGCACGTGCTGAGAGAACCCGACGGC
CysArgAsnProAspGlyHisPheAlaLysProTrpCysHisValLeuLysAsnArgArgLeu

961 971 981 991 1001 1011
TGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGGGCTGAGACAGTACAGCC
ThrTrpGluTyrCysAspValProSerCysSerThrCysGlyLeuArgGlnTyrSerGln

1021 1031 1041 1051 1061 1071
AGCCTCAGTTTCCGATCAAGAGGGGCTCTTCCGCCAGATCGCCTCCACCCCTGGGAGG
ProGlnPheArgIleLysGluGlyLeuPheAlaAspIleAlaSerHisProTrpGlnAla

1081 1091 1101 1111 1121 1131
CTGCCATCTTTGCCAAGCAGAGGAGGTGCGCGGAGAGCGGTTCCTGTGCGGGGGCATAC
AlaIlePheAlaLysHisArgArgSerProGlyGluArgPheLeuGlyGlyIleLeu

1141 1151 1161 1171 1181 1191
TCATCAGCTCCTGCTGGATTCTCTCTGCGCGCCACTGCTTCAGAGAGAGGTTTCCGCCCC
IleSerSerCysTrpIleLeuSerAlaAlaHisCysPheGlnGluArgPheProProHis

1201 1211 1221 1231 1241 1251
AGCACCTGACCGTGTCTTGGCCAGAACATACCGGCTGGTCCCTGGCAGGAGGAGGAGCA
HisLeuThrValIleLeuGlyArgThrTyrArgValValProGlyGluGluGluGlnLys

1261 1271 1281 1291 1301 1311
AATTTCAAGTCGAAAAATACATTGTCCATAGCAATTCGATGATGACACTTACGACAAATC
PheGluValGluLysTyrIleValHisLysGluPheAspAspAspThrTyrAspAspAsp

1321 1331 1341 1351 1361 1371
ACATTGCGCTGCTGCAAGCTGAAATCGGATTGCTCCCGCTGTGCCAGGAGAGAGCGCTGG
IleAlaLeuLeuGlnLeuLysSerAspSerSerArgCysAlaGlnGluSerSerValVal

1381 1391 1401 1411 1421 1431
TCCGCACTGTGTGCTTCCCGCGGAGCTGCAAGCTGCCAGACTGGAGCGAGTGTAGCC
ArgThrValCysLeuProProAlaAspLeuGlnLeuProAspTrpThrGluCysGluLeu

1441 1451 1461 1471 1481 1491
TCTCCGGCTACGCCAAGCATGAGGCTTGTCTGCTTTCTATTCGGAGCGGCTGAGAGGAGC
SerGlyTyrGlyLysHisGluAlaLeuSerProPheTyrSerGluArgLeuLysGluAla

1501 1511 1521 1531 1541 1551
CTGATGTCAAGCTGTACCCATCCAGCCCTGCAATCAGAACATTTACTTAAACAGAACAG
HisValArgLeuTyrProSerSerArgCysThrSerGlnHisLeuLeuAsnArgThrVal

1561 1571 1581 1591 1601 1611
TCACCGACACATGCTGTGCTGTCGACAGACTGCGAGCGCGCGCGCGCGAGCAAACTTGC
ThrAspAsnHisLeuCysAlaGluAspThrArgSerGluGluProGlnAlaAsnLeuHis

1621 1631 1641 1651 1661 1671
ACGAGCGCTGCCAGCGCCGATTCCGGAGCGCCCTGCTGTCTGTGACCATGCCCGCATCA
AspAlaCysGlnGlyAspSerGlyGlyProLeuValCysLeuAsnAspGlyArgHisThr

1681 1691 1701 1711 1721 1731
CTTTGCGTGGCATCATCAGCTGGGGCTGGCTGTGGACAGAGGATGTCCCGGCTGTGT
LeuValGlyIleIleSerTrpGluLeuGlyCysGluGlnLysAspValProGluValTyr

1741 1751 1761 1771 1781 1791
ACACAAAGGTTACCAACTACCTAGATCGAATTCGTGACAAAGATCCGACCGTGACCAAGAA
ThrLysValThrAsnTyrLeuAspTrpIleArgAspAsnHisArgProTer

1801 1811 1821 1831
CACCGCACTGCTCAAAAGCAATGAGATCCGATCC

FIG. 9a

61102/86

61102/86

TRIS-KRINGLE PA OF FIG. (1b).

1	11	21	31	41	51	
CGATCCAGCAATCATGATGCAATGAGAGAGGGCTCTGCTGTGCTGCTGCTGCTGCTG						1PA
MetAspAlaMetLysArgGlyLeuCysCysValLeuLeuLeuCysGly						Signal peptide
61	71	81	91	101	111	30 a.a.
AGCAGTCTTCTGTTTCCGCCAGCCAGGAATCCATGCCCGATTCCAGAGAGGAGCCAGATC						
AlaValPheValSerProSerGlnGluIleHisAlaArgPheArgArgGlyAlaArgSer						
121	131	141	151	161	171	
TTACCHAGTGTATCTCCAGAGATCAAAAAACCCAGATGATATACCHAGCAACATCAGTCATG						
TyrGlnValIleCysArgArgGluLysThrGlnMetIleTyrGlnGlnHisGlnSerTrp						
181	191	201	211	221	231	
GCTGCCCGCTGTGCTCAGAGACACCGGCTGGATATTGCTGGTGCACAGTGGCAGGGC						
LeuArgProValLeuArgSerAsnArgValGluTyrCysTrpCysAsnSerGlyArgAla						
241	251	261	271	281	291	
ACAGTCCCACTCAGTGCCTGTCAAAAGTTGCGGAGCCAGGCTGTTTCAAGGGGGGAC						
GlnCysHisSerValProValLysSerCysSerGluProArgCysPheAsnGlyGlyThr						
301	311	321	331	341	351	
CTGCCAGCAGGCCCTGTACTTCTCAGATTTCTGTGTCAGTGGCCCGAGGATTTGCTGG						
CysGlnGlnAlaLeuTyrPheSerAspPheValCysGlnCysProGluGlyPheAlaGly						
361	371	381	391	401	411	
GAGTGTCTGTGAATAGATACAGGCCACGCTGCTATGAGCGGAATGCTCACTTTTACCG						
LysCysCysGluIleAspThrArgAlaThrCysTyrGluGlyAsnGlyHisPheTyrArg						
421	431	441	451	461	471	
AGGAAACCCAGCACTGACACCATGGCCCGGCTGCTGCTGCTGCTGCTGCTGCTGCTG						UK Kringle
GlyLysAlaSerThrAspThrMetGluArgProCysLeuProTrpAsnSerAlaThrVal						aa 50-131
481	491	501	511	521	531	
CCTTCAGCAAACTACCATGCCAGCAGATCTGATGCTCTTCAGCTGGCCCTGGGGAACA						
LeuGlnGlnThrTyrHisAlaHisArgSerAspAlaLeuGlnLeuGlyLeuGlyLysHis						
541	551	561	571	581	591	
TANTTACTGCAGCAACCCAGCAACCCGAGCCGACCTGCTGCTATGTCCAGGTGGCCCT						
AsnTyrCysArgAsnProAspAsnArgArgArgProTrpCysTyrValGlnValGlyLeu						
601	611	621	631	641	651	
AAAGCCGCTTGTCCAGCACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG						Hexapeptide
LysProIleValGlnGluCysMetValHisAspCysSerGluGlyAsnSerAspCysTyr						Linker
661	671	681	691	701	711	
CGAGCACCCAGCCATACCTACAGGGGACGTCGAGCAACGCGGAGAGTGGCGGCGAGTG						
GluArgGlnGlyIleSerTyrArgGlyThrTrpSerThrAlaGluSerGlyAlaGluCys						
721	731	741	751	761	771	
CHCCAACTCGAAGCAGCAGCGGCTTGGCCAGAGCCCTACAGCGGGCGGAGGCGCAGCCG						
ThrAsnTrpAsnSerSerAlaLeuAlaGlnLysProTyrSerGlyArgArgProAspAla						
781	791	801	811	821	831	
CATCAGGCTGGGCTGGGGAACCACTACTGCAAGAACCCAGATCGAGACTCAAGCC						
IleArgLeuGlyLeuGlyAsnHisAsnTyrCysArgAsnProAspArgAspSerLysPro						
841	851	861	871	881	891	
CTGCTGTACTGCTTTTAAAGCGGGAGTACAGCTCAGCTTTCAGCAACCCCTGCTG						
TrpCysTyrValPheLysAlaLysLysTyrSerSerGluPheCysSerThrProAlaCys						

FIG. 10

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

Example B (continued)

91 911 921 931 941 951
CTCTGAGGGAAACAGTGCAC TGC TACTTTGGCAATGGGTCAAGCTTACCGTGGCAGCAGCAG
SerGluGlyAsnSerAspCysTyrPheGlyAsnGlySerAlaTyrArgGlyThrHisSer

961 971 981 991 1001 1011
CCTCACCGAGTCGGGTGCCTCCTGCCCTCCCTGGCAATTCATGATCTCTGATAGCCAGGT
LeuThrGluSerGlyAlaSerCysLeuProTrpAsnSerMetIleLeuIleGlyLysVal

1021 1031 1041 1051 1061 1071
TTACACAGCAGACAGACCCAGTGCCTCAGGCCTCTGGGCTGGGCAACATAATTACTGCCG
TyrThrAlaGlnAsnProSerAlaGlnAlaLeuGlyLeuGlyLysHisAsnTyrCysArg

1081 1091 1101 1111 1121 1131
GAATCCTGATGGGGATGCCAAGCCCTGGTGGCAGCTGCTGAAGAACCGCAGGCTGACGTG
AsnProAspGlyAspAlaLysProTrpCysHisValLeuLysAsnArgArgLeuThrTrp

1141 1151 1161 1171 1181 1191
GGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCA
GluTyrCysAspValProSerCysSerThrCysGlyLeuArgGlnTyrSerGlnProGln

1201 1211 1221 1231 1241 1251
GTTTCGCATCAAAGGAGGGCTCTCTCGCCGACATCGCCTCCCGCCCTGGCAGGCTGCCAT
PheArgIleLysGlyGlyLeuPheAlaAspIleAlaSerHisProTrpGlnAlaAlaIle

1261 1271 1281 1291 1301 1311
CTTTGCCAAGCAGGAGGTGCGCCGGAGAGCGGGTTCTGTGCGGGGGCATACTCATCAG
PheAlaLysHisArgArgSerProGlyGluArgPheLeuCysGlyGlyIleLeuIleSer

1321 1331 1341 1351 1361 1371
TCTCTGCTGGATTCTCTCTCGCCGCGACCTGCTTCCAGGAGAGGTTTCCGCCCCACCCT
SerCysTrpIleLeuSerAlaAlaHisCysPheGlnGluArgPheProProHisHisLeu

1381 1391 1401 1411 1421 1431
GACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCAGGAGCAGCAGAAATTTGA
ThrValIleLeuGlyArgThrTyrArgValValProGlyGluGluGlnGlnPheGlu

1441 1451 1461 1471 1481 1491
AGTCGAAAAATACATTGTCCATANGGATTCGATGATGACACTTACGACAAATGACATTGC
ValGluLysTyrIleValHisLysGluPheAspAspAspThrTyrAspAsnAspIleAla

1501 1511 1521 1531 1541 1551
GCTGCTGCAGCTGAATTCGATTTCGTCGGCTGTGCCACGAGAGCAGGCTGGTCCGCAC
LeuLeuGlnLeuLysSerAspSerSerArgCysAlaGlnGluSerSerValValArgThr

1561 1571 1581 1591 1601 1611
TGTGTGCTTCCCGCGCGGCTCGACATGCTCGGACTCGACGGAGTGTGAGCTCTCCGG
ValCysLeuProProAlaAspLeuGlnLeuProAspTrpThrGluCysGluLeuSerGly

1621 1631 1641 1651 1661 1671
CTACGGCAGCATGAGGCTTGTCTCCTTTCTATTCCGAGCGGCTGAGGAGGCTCATGT
TyrGlyLysHisGluAlaLeuSerProPheTyrSerGluArgLeuLysGluAlaHisVal

1681 1691 1701 1711 1721 1731
CAGACTGTACCCATCCAGCCCTGCGACATCACACATTTACTTAAACAGAACAGTCAACCGA
ArgLeuTyrProSerSerArgCysThrSerGlnHisLeuLeuAsnArgThrValThrAsp

1741 1751 1761 1771 1781 1791
CAACATGCTGTGCTGGAGACATCGGAGCGCGCGGCCCGCCAGCCAACTTGACGACGGC
AsnMetLeuCysAlaGlyAspThrArgSerGlyGlyProGlnHisAsnLeuHisAspAla

1801 1811 1821 1831 1841 1851
CTGCCAGGCGGATTCGGAGGCGCCCTGGTGTGTCTGAACGATGCGCCGATGACTTTGGT
CysGlnGlyAspSerGlyGlyProLeuValCysLeuAsnAspGlyArgMetThrLeuVal

1861 1871 1881 1891 1901 1911
CGGCATCATCAGCTGGGCTGGGCTGTGCACAGAGGATCTCCCGGCTGTGTACACAA
GlyIleIleSerTrpGlyLeuGlyCysGlyGlnLysAspValProGlyValTyrThrLys

1921 1931 1941 1951 1961 1971
GGTTACCAACTACCTAGACTGGATTCTGACACACTGCGACCTGACCAAGGACACCGCA
ValThrAsnTyrLeuAspTrpIleArgAspAsnMetArgProTer

1981 1991 2001
CTCCTCAAAGCAAAATGAGATCCCGATCC

527

FIG. 10a

FIG. 10a

61102/86

Example C (continued)

901 911 921 931 941 951
CTCCAGGGGCACTCCGACTGCTATGAGGGCAATGGTCACTTTTACCGAGGAAGGCCAG
SerGluGlyAsnSerAspCysTyrGluGlyAsnGlyHisPheTyrArgGlyLysAlaSer Hexapeptide linker

961 971 981 991 1001 1011
CACTGACACCATGGGCGGCCCTGGCTGGCACTCTGCCACTGTCTTCAGCAAC
ThrAspThrMetGlyArgProCysLeuProTrpAsnSerAlaThrValLeuGlnGlnThr UK Kringle
a.a. 50-131

1021 1031 1041 1051 1061 1071
GTACCATGCCACAGATCTGATGCTCTTCAGCTGGGCCCTGGGGAACATATTACTGCAG
TyrHisAlaHisArgSerAspAlaLeuGlnLeuGlyLeuGlyLysHisAsnTyrCysArg

1081 1091 1101 1111 1121 1131
GAACCCAGACACCGGAGGCGACCCCTGGTCTATGTGCAGGTGGGCCCTAAGCCGCTTGT
AsnProAspAsnArgArgArgProTrpCysTyrValGlnValGlyLeuLysProLeuVal

1141 1151 1161 1171 1181 1191
CAGAGCTGATGCTGCATGACTGCTCCACTGCGGCCCTGACAGCTACAGCCAGCCTCA
GlnGluCysMetValHisAspCysSerThrCysGlyLeuArgGlnTyrSerGlnProGln

1201 1211 1221 1231 1241 1251
GTTTCGCATCAAGGAGGGCTCTTCGCCGACATCGCCTCCACCCCTGGCAGGCTGCCAT
PheArgIleLysGlyGlyLeuPheAlaAspIleAlaSerHisProTrpGlnAlaAlaIle

1261 1271 1281 1291 1301 1311
CTTTGCCAGCAGGAGGCTCGCCCGGAGAGCGGTTCTGTGCGGGGGATACTCATCAG
PheAlaLysHisArgArgSerProGlyGluArgPheLeuCysGlyGlyIleLeuIleSer

1321 1331 1341 1351 1361 1371
TCTCTGCTGGATTCTCTGCGGCCACTGCTTCCAGGAGAGGTTTCCGCCCCACACCT
SerCysTrpIleLeuSerAlaAlaHisCysPheGlnGluArgPheProProHisHisLeu

1381 1391 1401 1411 1421 1431
GACGGTGATCTTGGCAGACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGA
ThrValIleLeuGlyArgThrTyrArgValValProGlyGluGluGluGlnLysPheGlu

1441 1451 1461 1471 1481 1491
AGTCGAAATATACATTGCTCATAGGAATTCGATGATGACACTTACGACATGACATTGC
ValGluLysTyrIleValHisLysGluPheAspAspAspThrTyrAspAsnAspIleAla

1501 1511 1521 1531 1541 1551
GCTGCTGCAGCTGAATCGGATTGGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCAC
LeuLeuGlnLeuLysSerAspSerSerArgCysAlaGlnGluSerSerValValArgThr

1561 1571 1581 1591 1601 1611
TGTGTGCTTCCCGCGGACCTGCAGCTGCGGACTGGACGGAGTGTGAGCTCTCCGG
ValCysLeuProProAlaAspLeuGlnLeuProAspTrpThrGluCysGluLeuSerGly

1621 1631 1641 1651 1661 1671
CTACGGCAGCATGAGGCCCTTGTCTCTTTCTATTCCGAGCGGCTGAGGAGGCTCATGT
TyrGlyLysHisGluAlaLeuSerProPheTyrSerGluArgLeuLysGluAlaHisVal

1681 1691 1701 1711 1721 1731
CAGACTGTACCATCCAGCCGCTGCACATCACACATTTACTTACAGACAGTCACCGA
ArgLeuTyrProSerSerArgCysThrSerGlnHisLeuLeuAsnArgThrValThrAsp

1741 1751 1761 1771 1781 1791
CAACATGCTGTGCTGCGAGCACTCGGAGCGGGGGCCCCAGGCAACTTGCACGAGCGC
AsnMetLeuCysAlaGlyAspThrArgSerGlyGlyProGlnAlaAsnLeuIleAspAla

1801 1811 1821 1831 1841 1851
CTGCCAGGGCGATTCCGGAGGCCCTGCTGTGTCTGACGATGGCCGATGACTTTTGGT
CysGlnGlyAspSerGlyGlyProLeuValCysLeuAsnAspGlyArgMetThrLeuVal

1861 1871 1881 1891 1901 1911
GGGCATCATCAGCTGGGCCCTGGGCTGTGCACAGAGGATGTCCCGGCTGTACACAAA
GlyIleIleSerTrpGlyLeuGlyCysGlyGlnLysAspValProGlyValTyrThrLys

1921 1931 1941 1951 1961 1971
GGTTACCAACTACCTAGACTGATTCGTGACACATGCCAGCGTGACAGGACACCCGA
ValThrAsnTyrLeuAspTrpIleArgAspAsnMetArgProTER

1981 1991 2001
CTCCTCAAAAGCAATGAGATCCGGATCC

FIG. 11a

2017 09 09 11:09

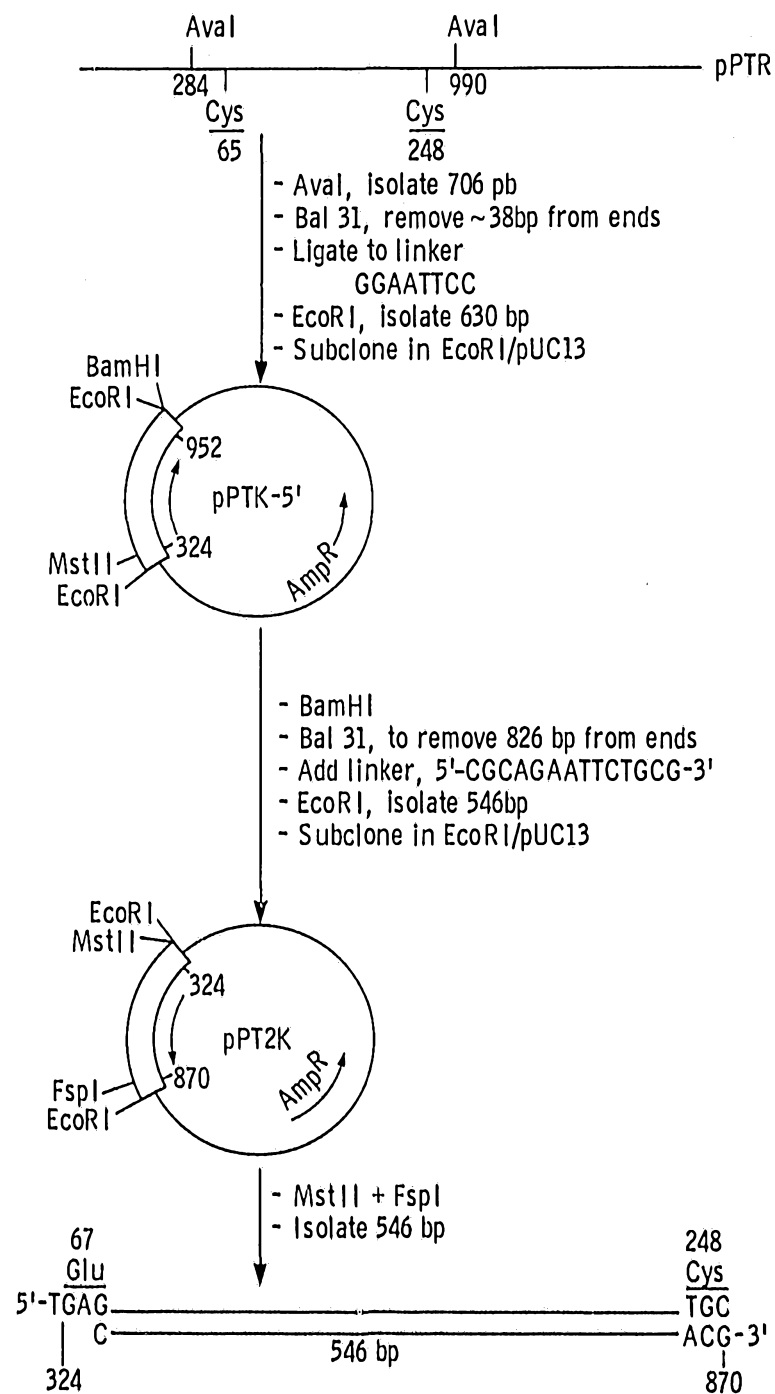


FIG. 12

5' 3' 5' 3'

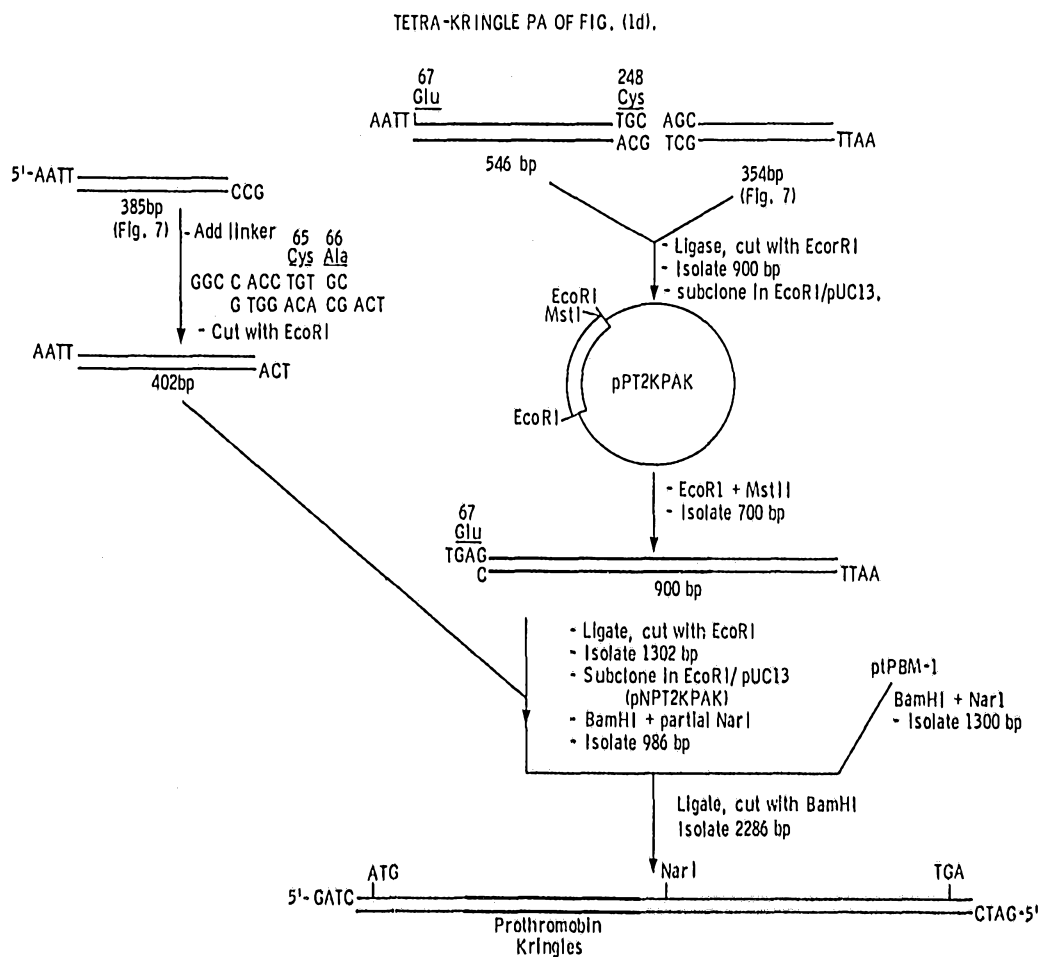
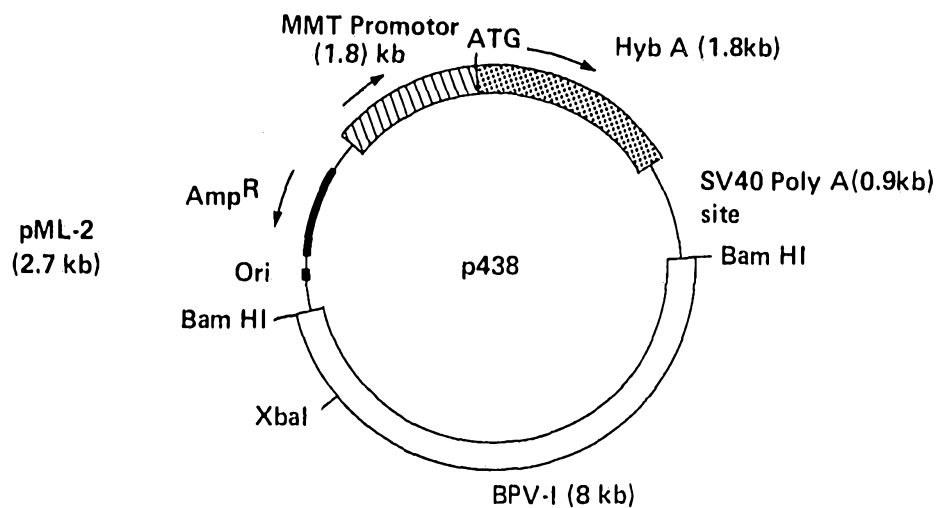


FIG. 13

A. EXPRESSION VECTOR: Hybrid A



B. EXPRESSION VECTOR: Hybrid B

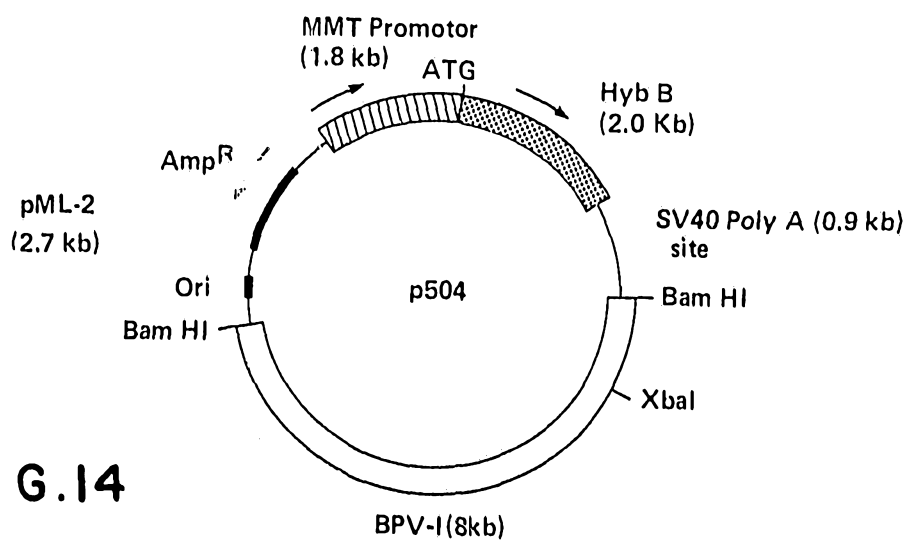


FIG.14

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FIG. 15

